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TARTRATE RESISTANT ACID PHOSPHATASE 5A: A POTENTIAL REGULATOR OF ADIPOCYTE CELL NUMBER AND DIFFERENTIATION IN WHITE ADIPOSE TISSUE

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Cover: Confocal microscopy image of pre-adipocytes treated with TRAP 5a at 4°C and stained for TRAP 5a (red)

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Tartrate resistant acid phosphatase 5a: A potential regulator of adipocyte cell number and differentiation in white adipose tissue

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family

ABSTRACT

Tartrate- resistant acid phosphatase (TRAP) exists in two isoforms, TRAP 5a which is monomeric and TRAP 5b which is a dimer generated by proteolytic cleavage of TRAP 5a, that exhibit different functions and localizations. TRAP 5a is expressed by adipose tissue macrophages and secreted into the extracellular environment and has been shown to lead to hyperplastic insulin- sensitive obesity when over-expressed in mice. In bone, TRAP is suggested to interact with the heparan sulfate proteoglycan (HSPG) glypican-4. In humans, TRAP 5a serum levels and TRAP in adipose tissue correlate to BMI. TRAP 5a has been shown to have a stimulatory effect on the proliferation and differentiation of pre-adipocytes, pre-osteoblasts and hematopoietic cells but the mechanism of action remains unknown. The aims of this thesis were (1) to develop an ELISA for quantification of human TRAP 5a and to evaluate TRAP 5a levels in lean vs. obese individuals and (2) to investigate the mechanism of action of TRAP 5a in pre-adipocytes and the effects on the homeostasis-related events such as cell cycle entry, differentiation and migration using the pre-adipocyte cell line 3T3-L1. A TRAP 5a ELISA was developed by generation of monoclonal antibodies specific for TRAP 5a and used to measure TRAP 5a in serum from lean and obese females. TRAP 5a in serum of obese females was reduced compared to lean but a positive correlation between BMI >30 and serum levels of TRAP 5a was identified. Regarding effect of TRAP 5a on 3T3-L1 cells, TRAP 5a was shown to bind to the components of extracellular matrix (ECM) heparan sulfate and nidogen-2. TRAP 5a was shown to bind to the pre-adipocyte cell membrane and to co-localize with HSPG glypican-4. After cell surface binding, TRAP 5a is endocytosed in a caveolae-mediated manner and co-localizes with early endosomal marker Rab-7. TRAP 5a was also observed in multivesicular bodies but did not co-localize with lysosomal marker LAMP-1. Mesenchymal stem cells, fibroblasts and pre-osteoblasts were also tested for their ability to bind TRAP 5a and only pre-osteoblasts exhibited TRAP 5a endocytosis. TRAP 5a was shown to enhance the number of cells entering S phase after cell cycle arrest by serum deprivation and to increase cyclin D1 levels. In a phosphorylation signaling array for proteins of the Akt pathway it was shown that TRAP 5a causes increased inactivation phosphorylation of GSK3β that is most likely not dependent on Akt since Akt activation phosphorylation was reduced after TRAP 5a treatment. Proteins controlling cell cycle arrest and apoptosis p53, p38 and Bad exhibited altered phosphorylation pattern after TRAP 5a treatment that could potentially lead to their inactivation. TRAP 5a treatment induced changes in morphology of pre-adipocytes and enhanced their migration. Focal adhesion protein (FAK), paxillin and actin were down-regulated after TRAP treatment indicating cytoskeletal changes and changes in the adhesion pattern. TRAP 5a treatment lead to enhanced lipid droplet acquisition after 2 days of differentiation induction in pre-adipocytes. In summary, TRAP 5a interacts with components of the ECM, binds to the surface of pre-adipocytes and co-localizes with glypican-4 and is endocytosed in a caveolae -dependent manner. Treatment of pre-adipocytes with TRAP 5a leads to enhanced cell numbers, increased migration, morphological changes and increased differentiation, all consistent with a growth factor-like role for TRAP 5a in adipose tissue.

LIST OF SCIENTIFIC PAPERS

I. **Christina Patlaka**, Staffan Paulie, Laia Mira Pascual,Peter Arner, Pernilla Lång, Göran Andersson. Development of a human tartrate resistant acid phosphatase 5a specific ELISA. Manuscript

II. **Christina Patlaka**, Hong Anh Mai, Pernilla Lång, Göran Andersson. The growth factor-like adipokine tartrate -resistant acid phosphatase 5a interacts with the rod G3 domain of adipocyte - produced nidogen-2. Biochem Biophys Res Commun., 2014, 454, 3, 446-452

III. **Christina Patlaka**, Heike Becker, Maria Norgård, Staffan Paulie, Annica Nordvall-Bodell, Pernilla Lång, Göran Andersson. Caveolae-mediated endocytosis of the glucosaminoglycan- interacting adipokine tartrate resistant acid phosphatase 5a in adipocyte progenitor lineage cells. Biochim Biophys Acta. 2014 Mar;1843(3):495-507.

IV. **Christina Patlaka**, Laia Mira Pascual, Pernilla Lång, Göran Andersson. Tartrate resistant acid phosphatase 5a regulates cell number and motility in 3T3-L1 pre-adipocytes. Manuscript

CONTENTS

LIST OF ABBREVIATIONS

aP2 Adipocyte protein 2

APPL Amyloid protein precursor-like

ATM Adipose tissue macrophages

BMI Body mass index

BMP Bone morphogenetic protein

C/EBPα CCAAT/enhancer binding protein α

cAMP Cyclic adenosine monophosphate

CCL Chemokine (C-C motif) ligand

Cdk Cyclin -dependent kinase

CHD7 Chromodomain-helicase-DNA-binding protein 7

COP-I Coat protein I

CXCL Chemokine (C-X-C motif) ligand

ECM Extracellular matrix

EEA Early endosomal antigen

EGF Epidermal growth factor

EPAC Exchange protein directly activated by cAMP

ER Endoplasmic reticulum

FAK Focal adhesion kinase

FFA Free fatty acid

FGF Fibroblast growth factor

GLUT-4 Glucose transporter type 4

GPCR G-protein coupled receptor

GSK3β Glycogen synthase kinase 3 β

GTP Guanosine-5'-triphosphate

HGF Hepatocyte growth factor

HSP27 Heat shock protein 27

HSPG Heparan sulfate proteoglycan

ICAM Intracellular adhesion molecular 1

IGF Insulin-like growth factor

IL-4 Interleukin- 4

JNK c-Jun N-terminal kinase

LAMP-1 Lysosomal-associated membrane protein 1

LRP Low-density lipoprotein receptor-related protein

MCP-1 Monocyte chemoattractant protein-1

MMP Matrix metalloproteinase

MSC Mesenchymal stem cells

mTOR Mechanistic target of rapamycin

MVB Multi-vesicular bodies

NLK Nemo-like kinase

PIK3 Phosphoinositide 3-kinase

PIP_2 Phosphatidylinositol 4,5-bisphosphate

PPARγ Peroxisome proliferator-activated receptor α

Pref-1 Pre-adipocyte factor 1

RAP1 Ras-proximate-1

Rb Retinoblastoma

RTK Receptor tyrosine kinases

SARA SMAD Anchor for Receptor Activation

SDF-1 Stromal cell-derived factor 1

SNARE SNAP Receptor

Stat 3 Signal transducer and activator of transcription 3

TG Triglycerides

TGF-β Tumor growth factor -β

TIMP3 Tissue inhibitor of metalloproteinases 3

TNF-α Tumor necrosis factor- α

VCAM Vascular cell-adhesion molecule 1

Wnt Wingless integration-1

1 INTRODUCTION

1.1 TISSUE HOMEOSTASIS

Tissue homeostasis refers to the maintenance of tissue in a functional state and is regulated by a variety of biological events regulating the life span of cells such as cell proliferation i.e. cell division, differentiation i.e. cell maturation, migration i.e. cell movement and cell death.

Tissues are multicellular structures comprised of different cell types. Tissue- specific cells are differentiated cells that have a particular function that can vary depending on the type of tissue e.g. a metabolic function like e.g. hepatocytes in liver or storage function (adipocytes in adipose tissue) or structural/barrier function such as keratinocytes in skin. Apart from differentiated cells, tissues contain progenitor/precursor cells, committed to a certain cell lineage, but not fully differentiated and retaining the ability, although reduced compared to e.g. stem cells, to proliferate (e.g. pre-adipocytes in adipose tissue). A fraction of stem cells named adult stem cells reside in tissue and serve as a reservoir pool for tissue regeneration of dying cells and maintain homeostasis in case of damage. Immune cells are additionally an important part of a tissue. Apart from dendritic cells and mast cells, resident macrophages are present in the stroma and are responsible for clearing of cells undergoing apoptosis and mediating the inflammatory response during tissue damage.

1.1.1 How is cell communication mediated?

Tissue homeostasis and the events described above (cell number regulation, cell migration and differentiation) are dependent on soluble signals from the extracellular environment (ECM), interaction of the cells with the ECM as well as with other cells.

ECM is involved in tissue homeostasis and cellular events in various ways. Growth factors can bind to the ECM and remain there until ECM degradation leads to their release and binding to the cells. Growth factors bind by the use of heparan sulfate (HS) of the ECM e.g. epidermal growth factor (EGF) and hepatocyte growth factor (HGF) and on heparan sulfate proteoglycans (HSPGs). Degradation of the ECM can lead to release of these factors once they are needed. Other growth factors, e.g. fibroblast growth factor (FGF) and transforming growth factor (TGF) use the proteoglycans (PG) of the ECM for binding to the cells, thus ECM facilitates the binding [1]. In other cases, the binding of growth factors to ECM serves a different purpose that is to make the growth factors available to the cells. Moreover, proteins of the ECM such as laminin contain intrinsic sequences (e.g. EGF-like domains) and have been suggested to directly bind to cell receptors and induce cell signalling [1].

Mesenchymal stem cell (MSC) commitment to a certain cell lineage is also dependent on factors of the ECM that will provide the appropriate signals for the cells to differentiate. Formation of adipocyte precursors (pre-adipocytes) is impaired by the presence of the ECM component fibronectin and the binding to its receptor integrin $\alpha5/\beta1$ [2]. This binding causes the levels of the Rho GTPase RAC to remain high, thus inhibiting adipocyte differentiation. Rho GTPases are important regulators of MSC commitment as they influence the shape of the cells and for e.g. adipocytes, changes in shape are crucial for differentiation [2]. The extracellular environment can also be altered by the activation of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) and influence cell lineage commitment, e.g. MMP14 and TIMP3 are involved in adipocyte lineage commitment [2].

1.1.2 Major endocytosis route for growth factors

Once bound to their receptors, growth factors can follow various routes to complete the downstream signalling. Endocytosis can lead to different outcomes, such as signal reduction by receptor degradation, downstream signalling by interaction with proteins of the endocytic vesicles or signalling enhancement by receptor recycling.

The main mechanism of growth factor endocytosis are the clathrin- dependent and clathrin - independent endocytosis (Figure 1). One of the most common mechanisms of clathrin-dependent endocytosis is via β-arrestins that bind to activated (phosphorylated due to ligand binding) receptors, e.g. G-protein coupled receptors (GPCRs) and clathrin and adaptor protein 2 (AP2) thus forming an endocytic complex [3].

The most common clathrin- independent endocytosis pathways is mediated via caveolae. Caveolin-1 interaction with cholesterol and phosphorylation of caveolin-1 are two of the mechanisms that are required for the formation of caveolae [4,5]. Both clathrin- and caveolae-mediated endocytosis require the presence of dynamin for the formation of clathrin- and caveolae-coated pits and for later invagination of vesicles by hydrolyzing guanosine-5'-triphosphate (GTP) [6,7]. Downstream endocytosis can follow different routes. The first step for both clathrin- and caveolae- mediated endocytosis is the formation of early endosomes, where signalling transduction can take place via components Rab5 together with early endosomal antigen (EEA) (canonical endosome signalling) or APPL by formation of complex with Protein kinase B (PKB) also known as Akt and Glycogen synthase kinase 3 (GSK3β) leading to its phosphorylation and downstream signalling [8]. After early endosomes, receptor signalling can follow alternative routes. Receptor can be transported back to the cell membrane via recycling endosomes that contain Rab-4 or Rab-11. Duration of signalling can be determined by the action of recycling endosomes [8]. Apart from recycling, receptors can be translocated to late endosomes and multivesicular endosomes/bodies for further transfer to the lysosomes for degradation but also for further signal enhancement. Late endosomes are characterized by the presence of Rab-7 that replaces the early endosome component Rab-5 [9].

For many growth factors, endocytosis takes place in order to sustain the signal by receptor recycling or in other cases to remove the ligand from the cell surface and transfer it to the lysosomes for degradation and signal reduction/deactivation. The endocytosis route can also be dependent on the ligand concentration. For example, EGF can use clathrin- dependent endocytosis for signal transduction at low concentrations but caveolae-dependent endocytosis for signal down-regulation at high concentrations [10]. For other types of signalling, e.g. wingless/integration-1 (wnt) signalling, endocytosis via clathrin can lead to signal down-regulation, while clathrin-independent endocytosis to sustainment of signalling [11].

Endocytosis can serve other purposes such as transmitting the signal from the cell membrane to specialized compartments where signalling is required. This is of particular importance in processes such as motility and migration of cells, where it has been shown that clathrin- and Rab5-mediated endocytosis is responsible for localized signalling of Rac that leads to actin re-organization and cell migration [12]. Finally, endocytosis alone can be determining for signal transduction, e.g. TGF-β binds to its receptor thus leading to its activation by phosphorylation and formation of endosomes, it causes the recruitment of SMAD2 by SMAD Anchor for Receptor Activation (SARA). This enables the phosphorylation of SMAD2 by TGFβ receptor (TGFβR) and its translocation to the nucleus for initiation of transcription [13,14]. Multivesicular bodies (MVB) are actively participating in and are vital for signalling. GSK3β signalling via Wnt is dependent upon endocytosis and MVB formation [15].

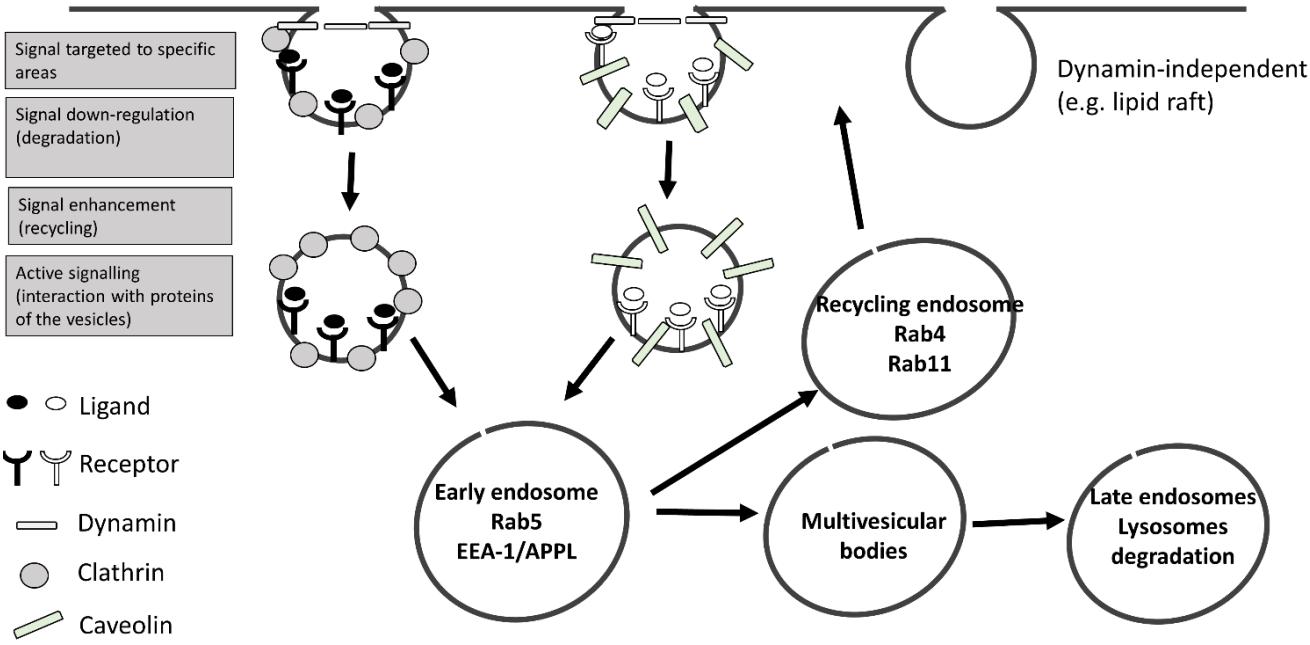

Figure 1. Endocytosis routes. Endocytic vesicles containing clathrin or caveolin and lipid rafts. After endocytosis there is formation of early endosomes that lead to recycling endosomes and transfer again to the membrane or to multivesicular bodies and late endosomes leading to lysosomal degradation.

1.1.2.1 Growth factor secretion from cells that regulate signals

Upon translation most growth factors and proteins follow conventional secretory pathways mediated via endoplasmic reticulum (ER)/golgi. Newly synthesized proteins can be transferred to ER and exit in coat-protein II (COP-II) vesicles to enter golgi and exit in coat-protein I (COP-I) coated vesicles that in their turn use SNAP Receptor (SNARE) complexes and a series of fusion of vesicles to exit through the cell membrane [16]. In the absence of internal signalling peptides that will guide them to the ER, proteins can follow alternative secretory pathways [17] that can bypass the golgi and be transmitted directly to the cell membrane or be mediated via caspase-1 [17]. Non-conventional secretory pathways can be determining for the protein's biochemical properties, e.g. FGF-2 follows ER/Golgi-independent secretion and when secreted via ER/golgi loses its ability to bind to HSPGs [18].

1.1.3 Cell number regulation

Tissue cell number is a balance between cell proliferation and cell death. In tissue, stem- and precursor cells reside without proliferating until a demand for new cells arises. Hence, these cells undergo transition from G1 to S phase by surpassing the restriction points in G1. This transition is regulated by a 'gas and break' system of signalling. The 'gas' will lead the cells to proliferation, while the 'break' will trigger (1) cell cycle arrest where cells will remain in G1 phase (also called G0) until further stimulation, (2) senescence where the cells will lose their ability to enter the cell cycle but will remain metabolically active or (3) cell death.

The gas in the transition of cells from G1 to S phase is the presence of mitogens which stimulate expression/activation of proteins needed for entering S phase by surpassing the G1/S restriction point. Mitogens such as EGF, insulin-like growth factor (IGF) and platelet-derived growth

factor (PDGF) are responsible for the primary signal. Mitogens bind to receptor tyrosine kinases (RTKs) causing their dimerization. [1920]. Activation of RTKs leads to phosphorylation and activation of Phosphoinositide 3-kinase (PIK3) that transiently activates Akt, thus causing inactivation of GSK3β by phosphorylation [20]. GSK3β when active phosphorylates β-catenin that will lead to its degradation before translocation to the nucleus, activation of E2F and initiation of transcription [21] (Figure 2). Additionally, GSK3β can regulate degradation of cyclin D1[22,23] that is vital for the transition from G1 to S phase [24]. Apart from Akt [25], GSK3β activity and β-catenin accumulation can also be regulated by the Wnt signalling pathway [26].Additionally after surpassing the restriction point controlled by Rb, transition from G1 to S phase requires the anchorage of cells which constitutes a second restriction point that is controlled independently of Rb [27].

Cell number is not only regulated by mitogens but also by cell cycle arrest, senescence and apoptosis signals that make up the 'break' in cell cycle progression. In the absence of mitogenic stimuli (e.g. in vitro during serum deprivation), cells will remain at the phase before the G1 restriction point (cell cycle arrest/G0) but will retain the ability to start proliferation and this phase is characterized by low levels of cyclins [28]. However, cells can also go into hypermitogenic cell cycle arrest that, unlike G0, is characterized by high levels of cyclins and cyclin dependent kinases (CDKs) and activated mitogenic pathways but also the activation of CDK inhibitors such as p21 and p16 [28]. Due to the activation of CDK inhibitors, evasion of hypermitogenic cell cycle arrest can occur not by the presence of mitogens but instead by activation of downstream targets such as E2F [28]. When ability for cell cycle progress is lost due to telomere shortage or by the presence of stress factor e.g. reactive oxygen species (ROS) or non-physiological conditions cells can undergo irreversible cell cycle arrest named senescence [28]. The response and molecular mechanisms controlling senescence vary depending on the stimuli [29], but in most cases, cell senescence involves p53 activation which in turn leads to the activation of CDK inhibitors p16, p21 and p15 [29,30] that inhibit the phosphorylation of pRb thus leaving it active and able to supress E2F [31]. Interestingly, unlike cells in cycle arrest, senescent cells remain metabolically active [29]. Cell cycle progress is dependent on inactivation or down-regulation of apoptotic signals from anti-oncogenic proteins. For instance p53, p38, c-myc and JNK need to be down-regulated for cell cycle progress [32,33], however c-myc has been shown to promote apoptosis or proliferation depending on the concentration of mitogens and its activity has been shown to be regulated also via the Wnt signalling pathway [34]. Apart from surpassing the restriction points, completion of cell cycle includes an increase in cell mass (cell growth). Mechanistic target of rapamycin (mTOR) is one of the main regulators of cell growth stimulation and its activity has also been associated with phosphorylation by Akt [20].

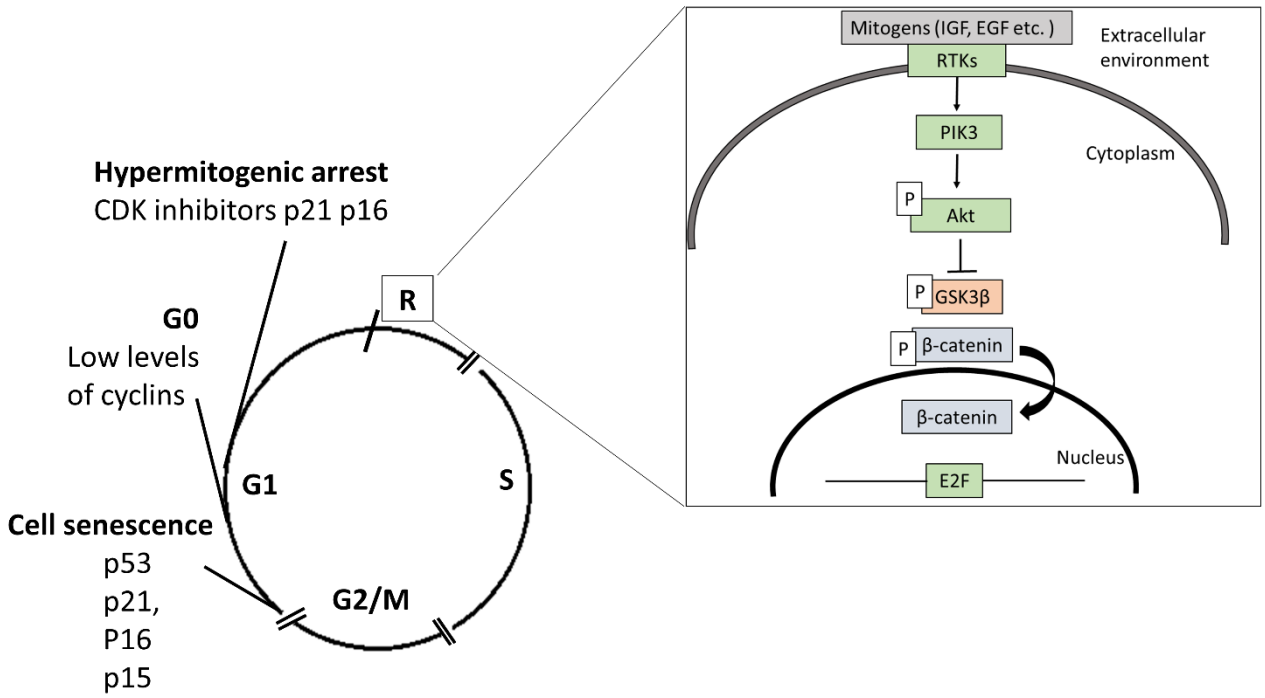

Figure 2. Cell cycle progression. Mitogens bind to RTKs leading to their activation. Downstream signalling activating PIK3 and Akt leads to de-activation of GSK3β and β-catenin translocation to the nucleus and transcription initiation. In the presence of high concentrations of mitogens, cells go into cell cycle arrest by expression of cdk inhibitors. Absence of mitogens leads to cell cycle arrest (G0). In the presence of stress or other stimuli cells lose their ability to enter the cell cycle and go into senescence controlled by p21, p16, p15 and p53. R= restriction point

1.1.4 Cell recruitment and migration

When there is need for tissue regeneration and /or tissue repair, recruitment of monocytes and stem cells from the blood stream to the tissue site is necessary. New cells need to migrate and infiltrate the tissue site where they proliferate and mature to differentiated cells in order to form new functional tissue. For recruitment, circulating cells in the blood need to be exposed and react to chemokines, adhere to and cross the endothelial barrier and finally move to the site where tissue needs to be repopulated.

Cells can be recruited in the presence or absence of an inflammatory signal. In the absence of inflammatory signals migration can be mediated via CXCL14 [35]. Inflammation -induced migration of monocytes is regulated via Chemokine (C-C motif) ligands (CCL) CCL2, CCL3 and Chemokine (C-X-C motif) ligand CX3CL1 [35]. Additional chemokines (e.g. CCL9) are responsible for their further recruitment to the lymph nodes. When chemokines bind to their receptors in the cells, they activate Ras-proximate-1 (RAP1) via cyclic adenosine monophosphate (cAMP) and exchange protein directly activated by cAMP (EPAC) which in turn binds to RALP. The RALP and RAP1 complex is associated to integrins and together with talin they induce morphological changes in the tails of α and β subunits of integrins giving them the ability to bind to intracellular adhesion molecular 1 (ICAM1) and vascular cell-adhesion molecule 1 (VCAM1) that are present on the surface of endothelial cells, thus inducing stronger adhesion. Integrins do not have activity potential on their own so they associate with other proteins and form complexes [36]. The C-terminal tails of integrins associate with focal adhesion

kinase (FAK) and paxillin which cause the complex in a phosphorylation-dependent manner to bind to vinculin and actin via talin. This complex leads to actin reorganization with migration and adhesion effects [36,37]. Moreover, activation of integrins leads to Rho GTPases activation that has an effect in actin re-organization. Integrin also causes up-regulation of lipid kinase activity and subsequent increase of Phosphatidylinositol 4,5-bisphosphate ($PIP_{2)}$ that lead to downstream signalling of Akt, Erk and c-Jun N-terminal kinase (JNK) and thus cause effects on proliferation, survival and differentiation. Additionally, increase in PIP_3 leads to cytoskeletal re-organization and changes in cell polarity.

Stem cells are also recruited to the tissue when there is a need for tissue repair or regeneration. Stem cell recruitment and migration is also mediated via chemokine, chemokine receptor and integrin activation as described above. Chemokine Stromal cell-derived factor 1 (SDF-1) and its receptor CXCR4 are the main mediators of stem cell migration, and SDF-1 is expressed in bone marrow leading to stem cells remaining there. When there is a need for stem cell recruitment to tissue, this tissue releases SDF-1 leading to recruitment of stem cells to the site where they are needed by the same downstream pathway as described for monocytes [38].

1.1.5 Cell differentiation

Once precursor/progenitor cells have reached the site where tissue repair or tissue regeneration is required, exposure to the microenvironment leads to their cell differentiation. Factors in the microenvironment, growth factors and their respective receptors can have a determining effect on the lineage commitment of cells and their subsequent differentiation. Mesenchymal stem cell commitment to a specific lineage e.g. bone or adipose is regulated by various signalling proteins such as wnts [39], bone morphogenetic proteins (BMPs) [40], but also by constituents that comprise the extracellular matrix (ECM)[41]. Wnt signalling occurs via pathways known as the canonical pathway and an alternative pathway named non-canonical pathway (Figure 3). Wnts in the canonical pathway (Wnt10b) act by binding to low-density lipoprotein receptor-related protein (LRP) co-receptors and frizzled receptor leading to structural changes in frizzled causing the binding and activation of Dishevelled and inhibition of GSK3β activity [42]. Canonical wnt signalling affects mesenchymal stem cells in various ways, resulting in inhibition or promotion of osteogenesis and adipogenesis depending on the level of maturation/differentiation of the mesenchymal stem cells [39].

Mesenchymal stem cells in early stages of their lineage differentiation remain proliferative and undifferentiated when canonical wnt signalling is active. In MSC committed to osteoblast lineage cells (pre-osteoblasts) but not mature cells, wnt10, wnt3a and wnt1 can promote differentiation by activating the transcription of Runx2 [11]. In committed adipocyte lineage cells (pre-adipocytes), canonical wnt signalling mainly by ligand wnt10b can inhibit differentiation by blocking CCAAT/enhancer binding protein α (C/EBPα) and PPARγ and the downstream protein adipocyte protein 2 (aP2)[43].

Non-canonical wnt pathways act independently of the frizzled receptor and can also mediate β-catenin accumulation or act independently of β-catenin (Figure 3). Wnt5a binding to other receptors than frizzled, also inhibits adipogenesis, by activating SETB1 that forms complex with chromodomain-helicase-DNA-binding protein 7 (CHD7) and nemo-like kinase NLK preventing peroxisome proliferator-activated receptor α (PPARγ) from transcribing adipocyte differentiation- related genes. wnt5b on the other hand, by binding to receptors other than frizzled, prevents β-catenin from translocating to the nucleus thus promoting adipocyte differentiation[43].

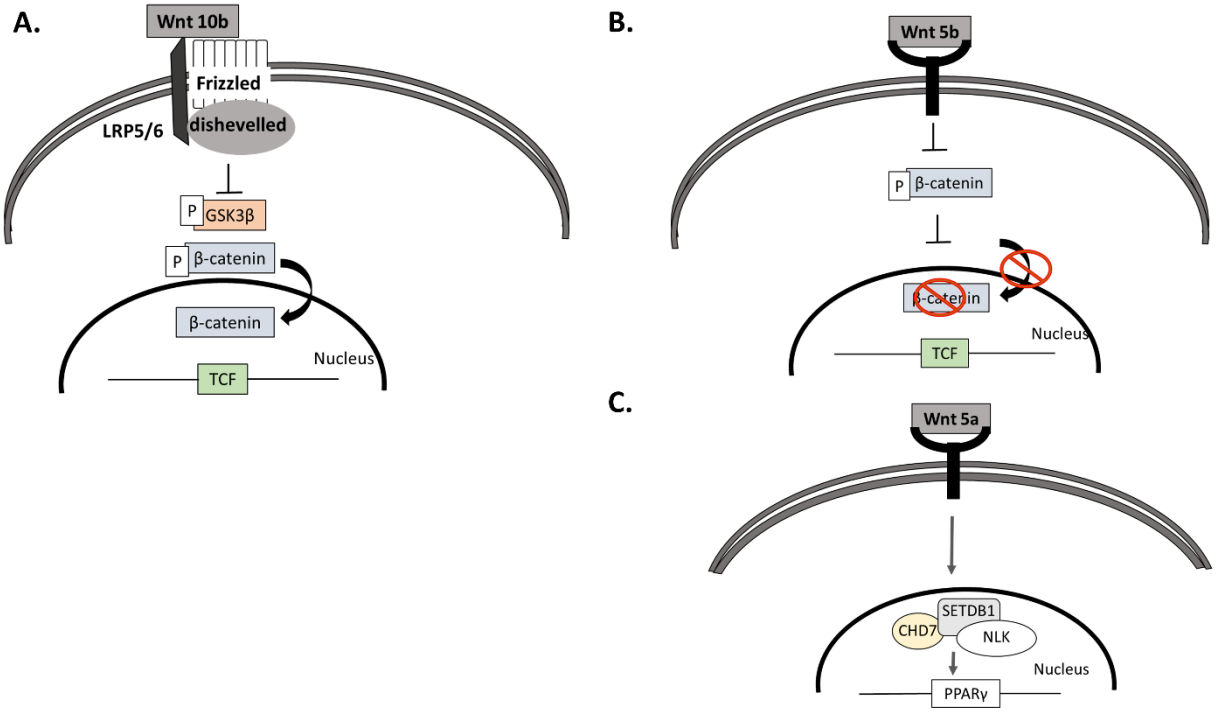

Figure 3. Wnt signalling pathways. A) Canonical Wnt signalling pathway. Wnt 10b binds to frizzled and LRP 5/6 leading to complex formation with dishevelled, inhibition of GSK3β and translocation of β-catenin to the nucleus. B) Non-canonical Wnt pathway, Wnt 5b binds to receptors that lead to inhibition of translocation of β-catenin to the nucleus and inhibition of TCF transcription. C) Non-canonical β-catenin independent pathway. Wnt 5a binds to receptors leading to complex formation of CHD7, SETDB1 and NLK and transcription of PPARγ

1.2 ADIPOSE TISSUE HOMEOSTASIS

1.2.1 Adipose tissue as an endocrine and metabolic organ

Adipose tissue is a metabolic organ responsible for energy uptake, storage of triglycerides (TG) and release of free fatty acids (FFA) [44], for hormone production and secretion and for secretion of adipokines that contribute to cross-talk with other organs and regulation of intermediary metabolism [45]. Hormones secreted from adipose tissue e.g. leptin have been shown to affect metabolism, whole body homeostasis and energy regulation [46–48].

1.2.1.1 Cell types in adipose tissue

Adipose tissue compromises mainly of adipocytes, the differentiated tissue specific cells that store fat. Adipocytes are characterized by the presence of one or several lipid droplet(s) that occupy the majority of the cell cytoplasm and which are controlled in size by the presence of perilipin [49]. Adipose tissue additionally contains adipocyte precursors (mesenchymal stem cells and pre-adipocytes) that reside in the tissue until tissue regeneration is needed, when they mature to fully differentiated adipocytes and resident macrophages that are involved in tissue homeostasis.

1.2.1.2 Main components of the ECM in adipose tissue

The ECM composition of adipose tissue plays an important role in adipocyte maturation and is necessary for the change in the cell-cell contact and change of the cell morphology during adipocyte differentiation [44]. During adipocyte differentiation, changes in the composition of ECM have been observed such as increases in collagen IV, laminin and nidogens [50–53]

1.2.1.3 Cell number

Formation of adipose tissue occurs from mesoderm [54] during early stages of development. Interestingly, even though the number of adipocytes remains stable during adulthood there is a 10% annual turnover [55,56] of adipocytes, resulting to a need for adipocyte development from pre-adipocytes and mesenchymal stem cells. There are various different factors contributing to adipose tissue homeostasis and turnover, among which resident macrophages [57] and ECM components [44] appears to be of particular importance.

1.2.2 Mesenchymal stem cells

Adipocytes, the main cell type in adipose tissue are derived from mesenchymal stem cells. MSCs were first described by Friedstein et al. in 1966 [58] and are non-hematopoietic stem cells that have the ability to differentiate into various types of tissue such as muscle, cartilage, bone and adipose tissue (Figure 4) [59,60] .

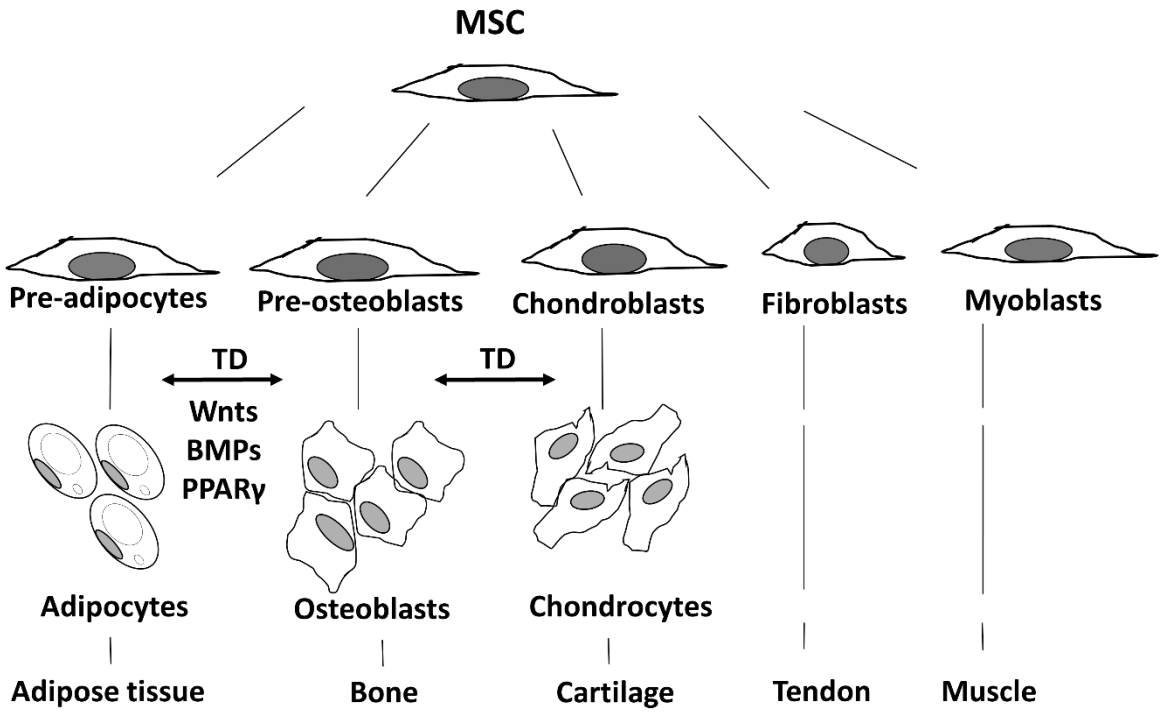

Figure 4. Mesenchymal stem cell lineage. Mesenchymal stem cell differentiation can lead to several different types of tissue. Trans-differentiation (TD) potential has been observed between adipocytes and osteoblasts and between chondrocytes and osteoblasts indicating a close relationship between those pre-cursors

Among the cell types that MSCs can differentiate to, pre-osteoblasts and pre-adipocytes are very close phenotypically and depend on the same signalling pathways that could inhibit differentiation towards one lineage and activate the other [39,61,62]. Wnt signalling, BMPs and PPARγ can guide the signalling towards adipogenesis or osteogenesis from MSCs, where e.g. wnt10b and wnt 5a lead the cells towards osteogenesis while wnt 5b leads the cells towards adipogenesis [2] and it has also been shown that differentiated cells can transdifferentiate from one cell type to the other, e.g. osteoblast can trans-differentiate to adipocytes [63,64] but also chondrocytes can trans-differentiate to osteoblasts [65,66].

1.2.3 From pre-adipocytes to adipocytes

Adipocyte differentiation has been studied extensively in vitro by the use of the murine fibroblast-like cell line 3T3-L1. In 1974, Meuth and Green described the ability of 3T3-L1 cells who have a fibroblast-like morphology to acquire lipid droplets and mature to adipocytes after stimulation with a hormone cocktail [67] and since then 3T3-L1 have been used as a model for both cell cycle and differentiation studies of pre-adipocytes to adipocytes. Pre-adipocyte to mature adipocyte progress includes various stages such as growth arrest, clonal expansion, early differentiation and terminal differentiation (Figure 5).

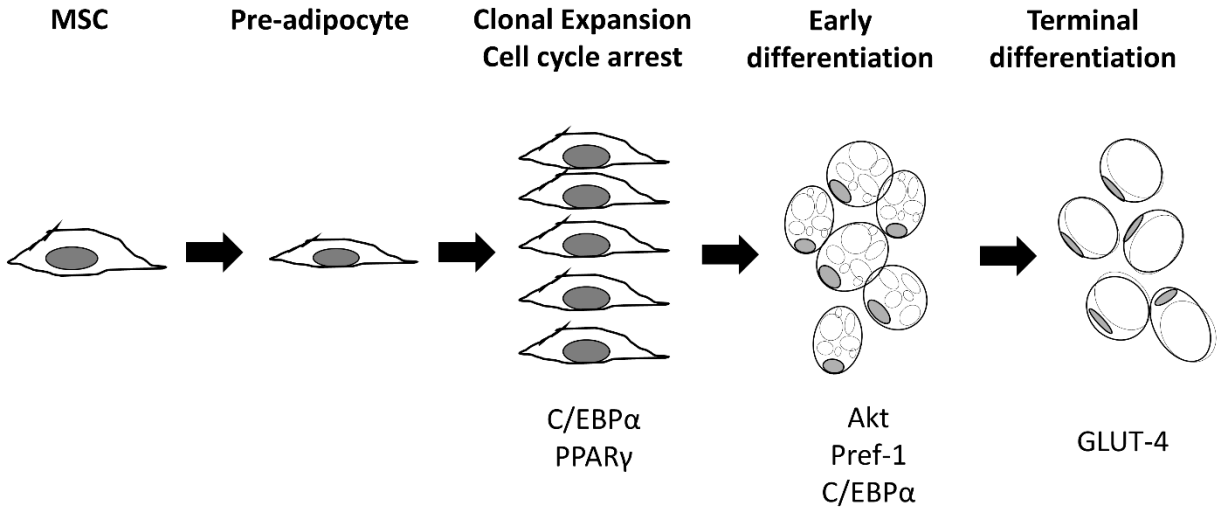

Figure 5. Adipocyte differentiation stages. Pre-adipocytes go into clonal expansion followed by growth arrest and characterized by increased levels of C/EBPα and PPARγ. Early differentiation is characterized by lipid acquisition, morphological changes, Akt signalling and Pref-1 down-regulation. Terminal differentiation is characterized by a merging of lipid droplets to one lipid droplet and expression of late markers e.g. GLUT-4.

1.2.3.1 Pre-adipocyte lineage

Characterization of cells as pre-adipocytes refers to cells that preserve the ability to proliferate, have a fibroblast-like morphology but are committed to the adipocyte cell lineage [2,68]. The main difference of pre-adipocytes to other fibroblast like cells that derive from mesenchymal stem cells is the presence of pre-adipocyte factor-1 (pref-1) protein. pref-1 is expressed in pre-adipocytes and its down-regulation is required for the initiation of adipocyte differentiation [69]. Pre-adipocytes reside in the adipose tissue and can be stimulated to proliferate and/or differentiate when it is needed and are retaining this capacity throughout life [70]. Tissue expansion starts with proliferation of pre-adipocytes that is followed by their differentiation to mature adipocytes. Pre-adipocytes are the principal cells in the adipose tissue that have a proliferative capacity and ability to differentiate into mature adipocytes [71,72].

1.2.3.2 Pre-adipocyte cell number regulation

Regulation of pre-adipocyte number is dependent on the control of the cell cycle. As with other cell types, G1 to S phase transition is controlled by growth factors that stimulate the downstream expression of CDKs. Insulin-like growth factor have been shown to participate in the stimulation of proliferation of pre-adipocytes [73] and also to induce resistance to stress factors and apoptosis in these cells. Insulin-like growth factor- binding proteins have also been reported to affect pre-adipocyte proliferation [74]. Finally hormones such as Thyroxins (T3, T4), glukocortikiods, adrenalin, noradrenalin have an impact on the metabolism of adipose tissue (lipogenesis and lipolysis).

1.2.3.3 Growth arrest

Once cells have reached confluence, cell-cell contact leads to cell cycle arrest. This process, however, has been shown not to be required for the differentiation of pre-adipocytes in vitro [44]. C/EBPα has been shown to regulate the cell growth arrest of pre-adipocytes by p21 mRNA and protein expression [75,76]. Transcription factor PPARγ can also induce cell growth arrest by reducing the DNA binding capacity of E2F/DP [77].

1.2.3.4 Clonal expansion

When differentiation is initiated, cell growth- arrested pre-adipocytes need to re-enter the cell cycle, at which stage they undergo proliferation known as clonal expansion. Interestingly it has been suggested that the molecular pathways for cell cycle proliferation before and after cell growth arrest (clonal expansion) differ [44]. Post- confluent pre-adipocyte clonal expansion is mediated via changes in the phosphorylation status of Rb and the transient increase of p107 [78]. However, cell cycle arrest that is required before early differentiation and is controlled by the expression of C/EBP [72] and is not mediated by Rb and Cdk inhibitors but can take place even when Rb is hypophosphorylated [79].

1.2.3.5 Early differentiation

Early differentiation stage of adipogenesis is characterized by the up-regulation of several transcription factors. Main regulators of adipocyte differentiation C/EBP and PPARγ are dramatically increasing during early differentiation stages. Moreover, the anti-adipogenic factor that is specific for pre-adipocytes, pref-1, is down-regulated at this stage. During early differentiation, cell morphology and ECM composition undergo important changes. More specifically, actin and tubulin are reduced [80], while there is a switch in the collagen types with an increase of collagen type IV and a decrease of type I and III procollagen and an increase in nidogen expression [51,81]. Akt signalling is essential for adipogenesis [82,83] and Akt knock-down

impairs expression of Krüppel-like factor that is required for completion of adipocyte differentiation [84,85].

1.2.3.6 Terminal differentiation

The final stages of adipocyte differentiation are characterized by rapid accumulation of lipids, mainly TG, fusion of small lipid droplets in to one large lipid vacuole that covers most of the cytoplasm of the cell. Terminal differentiation is also accompanied with increased insulin sensitivity and expression of perilipin, Glucose transporter type 4 (GLUT-4) and enzymes involved in triacylglycerol metabolism [44]. ECM re-organization has been shown to be essential for terminal differentiation and it has been shown that inhibition of collagen production impairs terminal adipocyte differentiation [86].

1.2.4 Macrophages of adipose tissue

1.2.4.1 Types of macrophages in adipose tissue

In adipose tissue the majority of immune cells are macrophages, also known as adipose tissue macrophages (ATMs). In lean adipose tissue the majority of ATMs are anti-inflammatory [87]. ATMs are generally classified into two groups, the pro-inflammatory cytokine secreting group M1 that is characterized by secretion of e.g. tumor necrosis factor α (TNF-α) that participate in the tissues immune defence and the anti-inflammatory cytokine secreting group M2 that secrete e.g. IL-4 that are regulating tissue homeostasis and can take part in tissue repair and remodelling [88,89]. ATMs can be recruited to the site by adipokines secreted from adipocytes [90] but it has also been shown that resident macrophages are able to proliferate by stimulation with IL-4 and do not need to be recruited from the blood stream [91]. In obesity, the ratio between anti-inflammatory (M2) and proinflammatory (M1) macrophages changes towards the majority of macrophages being polarized into M1 pro-inflammatory and this switch is triggered by secretion of adipokines from adipocytes as well as from an autocrine system from macrophages themselves [88,92–94].

1.2.4.2 Anti-inflammatory macrophages (M2)

Anti-inflammatory macrophages (M2) that reside in healthy adipose tissue, are activated by PPARγ and are positively regulating insulin-sensitivity [95]. Contrary to the popular belief that macrophages are hindering adipocyte differentiation and development it has also been shown that impairment of pro-inflammatory activation in mice leads to reduced capacity of tissue expansion [57] and it is known that M2 macrophages increase anti-inflammatory cytokines like IL-10 and participate in tissue repair [89] M2 macrophages can trigger tissue repair by the production of MMPs leading to ECM remodelling, growth factors and cytokines that trigger proliferation and differentiation of precursor cells in tissue[96] and are responsible for metabolic effects such as protection from insulin resistance via production of IL-10 [92,95].

1.2.4.3 Pro-inflammatory macrophages (M1)

On the other hand, M1 pro-inflammatory macrophages that are recruited and triggered by the expression of TNF-α, monocyte chemoattractant protein-1 (MCP-1) and adipokines are correlated to BMI and have been shown to contribute to the metabolic syndrome symptoms, tissue remodelling, and chronic inflammation [88,89,93,94,97,98].

1.2.5 Adipose tissue changes in obesity

Adipose tissue is now considered an important secretory organ for adipokines that influences the function of several organs [99], thus changes in tissue homeostasis or adipose tissue malfunctions leads to systemic problems. Obesity has been shown to be associated with an increase in the amount of ATMs [100] presumably due to increased death of adipocytes [101] where macrophages gather to remove the dead cells and hypoxia in the tissue [102] leading to chronic low grade inflammation [103]. Inflammation in adipose tissue has been shown as the cause of several conditions such as cardiovascular disease, atherosclerosis, type-2 diabetes and dyslipidemia that all together are referred to as the metabolic syndrome [104].

Adipose tissue expansion results in secretion of adipokines that will trigger the recruitment of macrophages and the 'phenotypic switch' of macrophages from anti-inflammatory M2 to pro-inflammatory M1 macrophages. Increase in pro-inflammatory M1 macrophages is mainly regulated by the MCP-1/CCR2 signalling that causes the recruitment of macrophages to adipose tissue [105,106]. MCP-1 and free fatty acid release (caused by lipolysis) are secreted by adipocytes that have become hypertrophic thus triggering the signalling for M1 macrophages [107] (Figure 6).

Obesity in adults is mainly characterized by the enlargement of the already existing adipocytes (hypertrophic obesity) and in some cases proliferation and increase in the adipocyte number (hyperplastic obesity) [108]. Hyperplasia is associated with better insulin response and glucose metabolism compared to hypertrophy in obesity [109]. Proliferation of pre-adipocytes occurs only after the full expansion of adipocytes [110] and cell growth of adipocytes leads to up-regulation and release of growth factors that can both promote the proliferation of pre-adipocytes e.g. IGF-I [111].

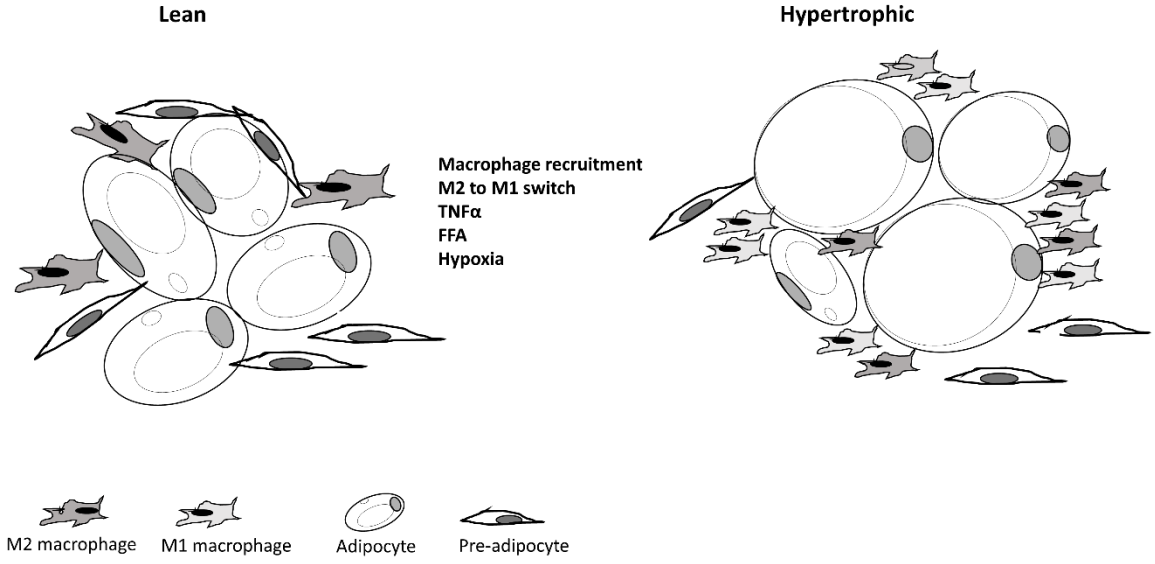

Figure 6. Adipose tissue remodelling in obese state. Increase of adipose tissue is characterized by increased TNFα, free fatty acids and hypoxia. These changes lead to macrophage recruitment and macrophage proliferation and morphological switch form anti-inflammatory m2 macrophages to pro-inflammatory M1 macrophages.

1.3 TARTRATE- RESISTANT ACID PHOSPHATASE

1.3.1 TRAP history and properties

Tartrate- resistant acid phosphatase (TRAP; E.C. 3.1.3.2), also known as purple acid phosphatase (PAP), AcP 5 or uteroferrin (Uf) [112,113] is a diiron centre- containing metalloenzyme [114,115]. TRAP was initially described as a member of acid phosphatases in 1954 by Sundararajan and Sarma [116] and acid phosphatase properties were investigated extensively in 1970s [117,118]. In 1971, Yam et al. described an isoenzyme of acid phosphatase that was tartrate- resistant and highly expressed in cells from the rare B-lymphocyte-derived hairy cell leukaemia [119]. During the 1970s and 1980s a variety of studies described common properties for purple acid phosphatase and tartrate- resistant acid phosphatase and in 1990s a study from Vincent and Averill proposed that purple acid phosphatase and tartrate resistant acid phosphatase were identical [113]. TRAP has traditionally been used as a marker for osteoclasts and activated macrophages [120–122], however in recent years more functions for TRAP have emerged.

1.3.1.1 TRAP gene and translation

The gene encoding TRAP (Acp5) is located on chromosome 19p13.2-13.3 in humans [123,124] and chromosome 9 in mouse [125] and contains five exons of which 4 are translated into protein sequences [126,127] while the untranslated exon 1 exists in 3 different variants in mouse (4 in humans) with giving rise to 3 mRNA transcripts encoding identical protein sequences but with differential cell- and tissue expression patterns [128–130].

1.3.1.2 Structure-proteolytic cleavage

TRAP exists in two different isoforms named TRAP 5a and TRAP 5b [131,132]. The product from translation of the Acp5 gene is the 35 kDa TRAP isoform denoted 5a that can undergo proteolytic cleavage giving rise to the dimeric isoform TRAP 5b of ~24 and 16 kDa "subunits", held together by a disulphide bond [133] (Figure 7) . Proteolytic cleavage of TRAP 5a occurs in a repressive loop region, Ser145-Val161[115,133] by various proteases, such as cathepsins, trypsin and papain and is responsible for the increased phosphatase activity of TRAP 5b [133–136]. The kinetic properties of TRAP are additionally influenced by the two N-linked oligosaccharide chains present in the protein [137].

Despite the fact that for many years isoform TRAP 5a was considered to serve only as the inactive precursor to the enzymatically active isoform TRAP5b, it has recently been apparent from several studies that the two isoforms can indeed have different functions and localizations and that TRAP 5a has a separate function promoting pre-adipocyte proliferation and differentiation and when over-expressed in mice leads to hyperplastic insulin sensitive obesity [138].

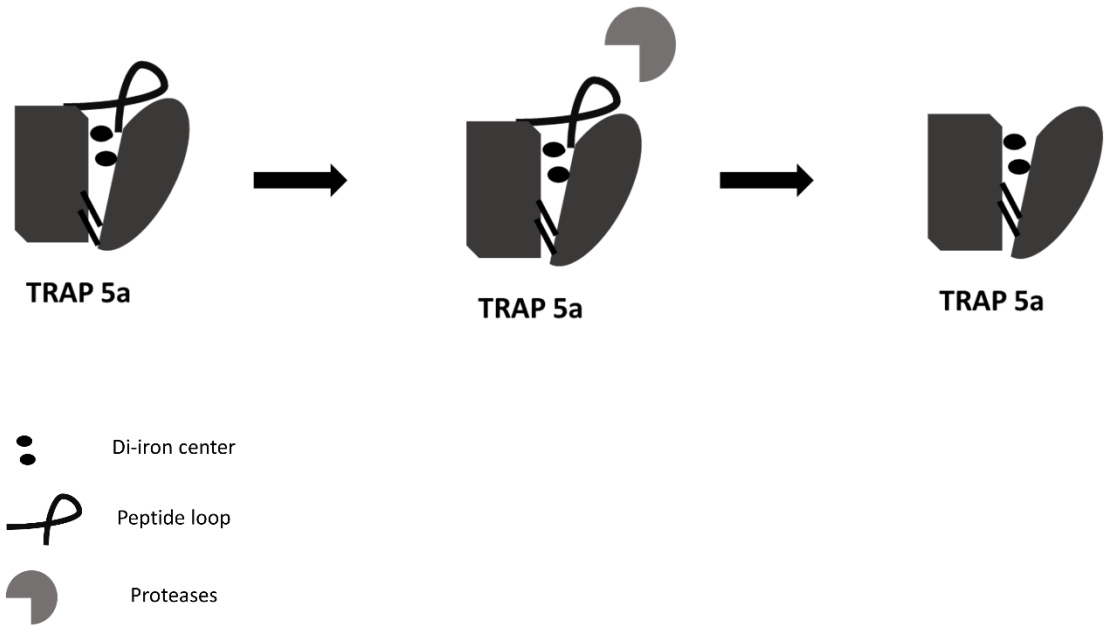

Figure 7. Proteolytic cleavage of TRAP 5a generates TRAP 5b. TRAP 5a is cleaved by proteases in a repressive loop that generates the "dimer" TRAP 5a held together by disulphide bonds and leading to changes in the di-iron centre increasing the phosphatase activity of the protein.

1.3.2 TRAP expression and localization

1.3.2.1 TRAP in macrophages and dendritic cells

TRAP is expressed by a subset of macrophages [139] [140] and dendritic cells [141] and correlates to their activation or maturational state. When TRAP is genetically knocked down in mice, an increased basal expression of inflammatory cytokines such as IL-1b, IL-12 and TNFα was noted[142] while dendritic cells in mice lacking TRAP have increased expression of IL-10 but reduced response to T- dependent antigens [143]. The expression of TRAP is also regulated by cytokines and it has been observed that TRAP is reduced in response to IFNγ and LPS [144] but another study indicated increased TRAP expression by LPS [145] and additionally TRAP expression is increased by TNFα in monocytes [146]

1.3.2.2 TRAP in bone

In bone TRAP has been shown to be expressed and secreted by osteoclasts. More specifically, TRAP 5a is secreted to the extracellular matrix where it is subjected to proteolytic cleavage giving rise to TRAP 5b while intracellular TRAP is found as both isoforms TRAP 5a and TRAP 5b [133,147,148].

Apart from osteoclasts, TRAP in bone is expressed by osteoblasts and osteocytes and its expression is independent of the osteoclast activity [149–153] but is increased in rats with osteoporosis [154]. Additionally, when TRAP is over-expressed in mice there is increased bone mineral content and density that stems from increased osteoblast differentiation since resorption activity remains stable [155] indicating a function of osteoblast TRAP different than that of osteoclast TRAP. TRAP in osteoblasts is located in LAMP-1 positive vesicles thus exhibiting lysosomal localization [156].

Finally TRAP has been shown to be expressed in cartilage by hypertrophic chondrocytes [156]. This may explain the cartilage phenotype observed in TRAP-deficient mice where the growth plate displayed increased height and disorganized columns of chondrocytes [157]. TRAP expression from chondrocytes and osteoblasts is of interest since they share a common mesenchymal stem cell lineage and particularly since TRAP expression in cells of the same lineage such as pre-adipocytes is very low [158], indicating a regulation of TRAP expression potential during commitment to different lineages.

1.3.2.3 TRAP expression from other cell types

Apart from the immune system and bone where TRAP expression has been extensively studied, TRAP expression has been observed in other cell types such as neurons [159], hepatocytes and keratinocytes [160].

1.3.2.4 TRAP binding partners

TRAP has been shown to interact with heparin [148,161,162] and heparin has been shown to inhibit activity of TRAP 5a but not TRAP 5b [163] . TRAP has also been suggested to interact with the HPSG glypican-4 in bone [164] and with the TGFβ receptor interacting protein 1 (TRIP-1) [152,165,166]. In serum, TRAP has been shown to be associated to α2-macroglobulin in a calcium-dependent manner [167].

1.3.3 TRAP in disease

1.3.3.1 TRAP 5a vs. TRAP 5b as disease markers

TRAP 5a and TRAP 5b have been suggested to have different localizations and serve as markers to different pathological conditions, where TRAP 5b has been shown to be a marker for osteoclast number and bone resorption in serum [133120–122] and end-stage renal disease [168] and kidney failure. On the other hand TRAP 5a has been suggested as a marker for mostly inflammatory conditions [169], such as atherosclerosis [170], rheumatoid arthritis, [171,172] and sarcoidosis [173].

1.3.3.2 TRAP and autoimmunity

Besides expression TRAP in certain macrophages, also certain dendritic cells known to be involved in antigen presentation, have been shown to be express TRAP with a proposed role in dendritic cell maturation[142]. Knock-down of TRAP in mice and in humans with inactivating mutations in the TRAP gene show a similar deregulated immune phenotype with increased basal expression of Th1 cytokines such as Interferon-γ (IFNγ), TNFα and IL-1[142,174]. This aberrant immune response was coupled to increased phosphorylation of osteopontin, a protein with known function to control TH1/Th2 bias through dephosphorylation by TRAP [175–177].

1.3.3.3 TRAP in cancer

TRAP has also been shown to be elevated in melanoma, gastric, colon, ovarian and breast cancer [178–183] and when over-expressed in a human breast cancer cell line, TRAP 5a and TRAP 5b can be found intracellularly while only TRAP 5a is secreted [184]. Moreover, TRAP has been shown to increase invasion and metastasis of cancer cells, to increase their migration and change their morphology and adhesion pattern [185]. In contrast, siRNA transfection revealed a morphological effect consistent with impaired detachment to plastic-bound substrates [186].

1.3.4 Regulation of cell number and differentiation by TRAP

TRAP has been shown to have effects on cell proliferation and differentiation [152,155]. In bone tissue TRAP has been shown to give rise to increased cortical bone mineral density and content and increased expression of bone differentiation markers when over-expressed [155] in mice and to have an effect on differentiation of osteoblasts [165,187] and to interact with the TGF-β-receptor-interacting protein (TRIP-1) that exerts a TGF-β like differentiation [152,166]. Osteoblast-like cells have also exhibited the ability to both express and endocytose TRAP [151,153] and TRAP levels are altered during osteoblast differentiation [187]. However, the exact function of TRAP expression and endocytosis by osteoblast-like cells in bone is not known. TRAP has additionally been shown to have an effect on cell number regulation of hematopoietic cells and to be a regulator of fetal erythropoiesis by acting synergistically with erythropoietin and other growth factors [188,189].

1.3.5 Regulation of migration by TRAP

Apart from cell number regulation TRAP has been shown to affect the motility, morphology and migration of cancer cells [185]. In osteoclasts, TRAP exhibits phosphatase activity with osteopontin as substrate [175,176] and dephosphorylation of osteopontin leads to reduced attachment of osteoclasts and changes in migration [190,191].

1.3.6 TRAP 5a in adipose tissue

Interestingly, isoform TRAP 5a has been shown to be involved in the regulation of the adipose tissue. In 2008, Lång et al. showed that when TRAP 5a is overexpressed in mice, it leads to hyperplastic insulin- sensitive obesity and that human and mouse pre-adipocytes show increased proliferation and differentiation by the addition of TRAP 5a but not 5b [138]. Additionally, no signs of inflammation were observed in the TRAP 5a over-expressing mice [138].

In adipose tissue, TRAP 5a has been shown to be elevated in obese individuals [192] and an increase of TRAP 5a has been described in the serum of overweight young males [193]. However, unlike other inflammatory markers, its expression was not reduced after exercise and weight loss [193]. TRAP expression in different adipose tissue depots also changes with obesity. In lean individuals TRAP levels are higher in visceral, while in overweight and obese individuals, higher levels of TRAP are observed in subcutaneous fat depot [98]. TRAP 5a expression and secretion in the adipose tissue is almost exclusively from macrophages [138,192] and even though TRAP 5a and TRAP 5b are both found intracellularly in ATMs, TRAP 5a is the only isoform that gets secreted [192]. Additionally, TRAP levels co-related to both number and size of adipocytes as well as with TNFα and IL-6 [192].

TRAP 5a secretion by macrophage suggests a paracrine action where TRAP 5a is secreted by macrophages but can act on pre-adipocytes and affect their proliferation and differentiation. However, until now little is known on the mechanism of action of TRAP 5a on cells in order to regulate cell number, differentiation and migration as well as its binding partners on the extracellular environment and cells.

1.3.7 Working hypothesis

The working hypothesis of this thesis was that TRAP 5a is secreted by a subset of macrophages in the adipose tissue, where it is presented to pre-adipocytes for binding to the cell surface and initiation of signalling pathways that can promote proliferation, differentiation and further effects in pre-adipocytes (Figure 8). However the molecular pathways, binding partners and secretion patterns of TRAP 5a in adipose tissue remain to be explained. In this thesis, the aim was to shed light on different aspects of TRAP 5a action in adipose tissue such as expression levels related to BMI, interaction of TRAP 5a with binding partners in ECM and cell surface, signalling pathways and functional effects in pre-adipocytes.

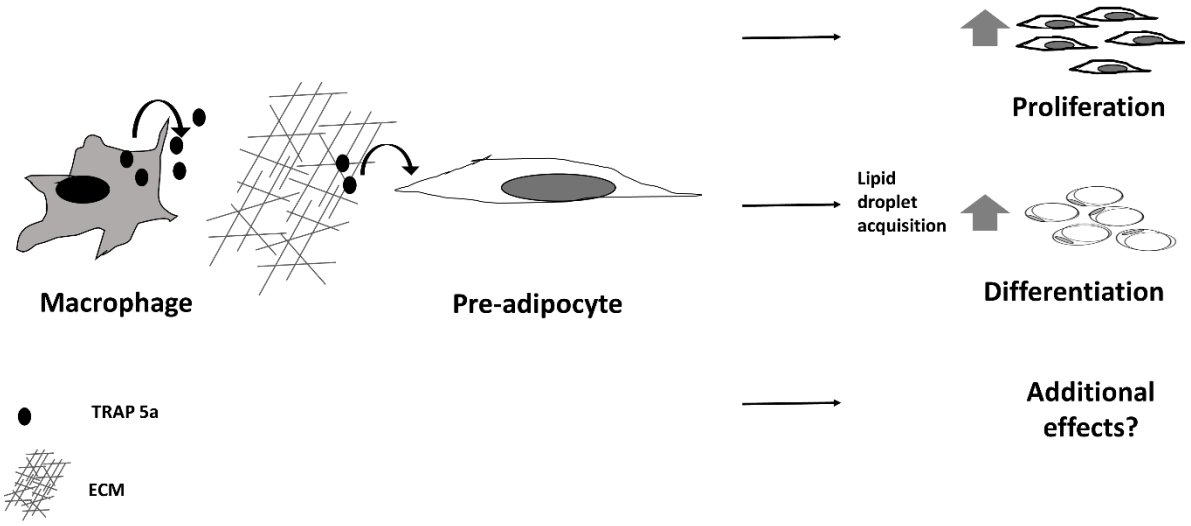

Figure 8. Working hypothesis of TRAP 5a mechanism of action in adipose tissue. TRAP 5a secreted from macrophages acts on pre-adipocytes in order to stimulate proliferation and differentiation a well as possible unknown effects.

2 AIMS OF THE THESIS

Paper I. The aim was to develop a human TRAP 5a specific ELISA in order to evaluate levels of TRAP 5a from obese vs. lean individuals.

Paper II. The aim was to identify potential binding partners of TRAP 5a in adipose tissue.

Paper III. The aim was to investigate the interaction between TRAP 5a and pre-adipocytes, the binding mechanism on the cell surface, endocytosis routes and interaction with other cell types of mesenchymal lineage.

Paper IV. The aim was to investigate the effect of TRAP 5a in the regulation of pre-adipocyte cell number, differentiation and migration and identify potential signalling pathways through which TRAP 5a action occurs.

3 REMARKS ON METHODOLOGY

3.1 TRAP 5A ELISA DEVELOPMENT

For ELISA development, the first step was the generation of monoclonal antibodies specific for human TRAP 5a. To achieve this, the peptide loop region present on human TRAP 5a but not human TRAP 5b was used as the immunogen by which mice were immunized. Apart from TRAP 5a antibodies, antibodies against total TRAP (TRAP 5a+TRAP 5b) were developed. To achieve this, the full length protein was used. Next, isolated antibodies were epitope mapped by ELISA. Epitope mapping of antibodies allows the grouping of antibodies for further use in a sandwich ELISA. The use of antibodies from different epitopes increases the possibility of both capture and detection antibodies to bind to the protein with high affinity and no interference of binding from one to the other.

One aspect of the quality assessment of the newly developed ELISA was the effect that serum components can possibly have on the detection of TRAP 5a. For this purpose recombinant TRAP 5a was diluted in serum in a dose- dependent manner and absorbance was measured.

The sandwich ELISA was evaluated by measuring absorbance in a TRAP 5a dose response, testing of TRAP 5b absorbance and measuring intra- and inter- assay variation and reference interval in a set of human serum samples from healthy males to verify the reproducibility of the results.

3.2 PULL DOWN ASSAYS

In paper II, the potential binding of TRAP 5a to nidogen-2 and TRIP-1 were studied in vitro using recombinant proteins in pull- down assays. In this system, His-tagged proteins were used as bait. The property of the His-tag to bind to Ni was used to bind the 'bait' proteins in magnetic agarose- Ni beads that could then be pulled-down by magnet. Ni- beads often exhibit considerable unspecific binding of proteins and a way to address this is saturation of beads with His-peptides to be used as control. In the pull- down assays described, control beads (beads with no His-tagged recombinant protein) were saturated with His- peptides to prevent unspecific binding.

One other important aspect to be taken into account when performing pull- down assays was the stoichiometry between the recombinant proteins that are studied. In the pull-down assays between His-tagged nidogen-2 domains and TRAP 5a, stoichiometry was always 1:1 for 'bait' and protein of interest.

3.3 ICC/ 2-STEP INDIRECT STAINING

To investigate if TRAP 5a can bind to the surface of pre-adipocytes, immunocytochemistry and indirect staining was used, where cells were treated with TRAP 5a for 1h and then fixed with formalin or aceton. After fixation cells were incubated with primary antibody against TRAP and fluorescent secondary antibody. The staining observed reflected only bound TRAP 5a since no TRAP 5a was detected in untreated cells. Since the potential binding partner for TRAP 5a was unidentified, to study binding of TRAP 5a cells were treated with TRAP 5a at 4°C to minimize endocytosis, thus ensuring that TRAP staining will only reflect TRAP 5a

bound to the surface. For the same purpose immunocytochemistry was performed without cell membrane permeabilization. Immunocytochemistry was followed by image analysis.

3.4 IMAGE ANALYSIS

In paper II, immunocytochemistry, proximity ligation assay and immunohistochemistry was used to evaluate nidogen-2 expression, interaction of TRAP 5a with nidogen-2 and TRIP-1 and co-localization of TRAP 5a with nidogen-2 in adipose tissue, respectively.

Image analysis was performed as follows: For proximity ligation assays the number of positive spots (each spots corresponds to an interaction site) were counted using Volocity software and divided with the area of each cell. For immunohistochemistry, co-localization was also quantified and analysed. In this case, co-localization was measured using Volocity software to calculate Pearson's correlation between the two colours (green for TRAP 5a and red for nidogen-2) as well as overlap coefficient M1 and M2 for the two channels, in five regions of interest. An important aspect of image analysis in order to generate Pearson's correlation and M1 and M2 values is setting threshold for signal. In all image analysis threshold was set by negative control of one of the two proteins, in order to minimize false positive co-localization. In adipose tissue, section that have not been stained for nidogen-2 served as negative control for threshold setting.

Overlap co-efficient refers to the amount of overlap of one channel towards the other, for instance M1 overlap coefficient refers to the amount of overlap of green channel to the red channel, while M2 refers to the overlap of red channel to the green channel. This is important in order to identify if two proteins are always co-localized (high M1 and M2 values) or if only one protein is always co-localized to the other (high M1 value but low M2 or vice versa) but the other one can be also observed in other areas.

Image analysis was also performed in immunocytochemistry and co-localization studies (paper III) following the same principles as described above. Since these studies were performed using pre-adipocytes, threshold was set for pre-adipocytes that had not been treated with TRAP 5a. Pre-adipocytes do not express TRAP 5a thus if not treated with TRAP 5a, no staining should be observed. This property can thus serve as a negative control. In this paper, quantification of ICC was also performed using velocity software and quantifying the number of clusters/area. For the quantification of clusters, parameters such as circularity, size and intensity of spots/clusters were standardized using TRAP 5a treated cells as a positive control. Data collected in the above method were statistically analysed in 4-10 cells using non-parametric Mann-Whitney U-test.

3.5 CELL CYCLE ANALYSIS

In paper IV, proliferation was studied by cell cycle analysis using the Bromodeoxyuridine (BrdU) incorporation method in combination with FACS analysis. This was performed in cells that were allowed to enter the cell cycle and left for various time points to progress in the cell cycle before BrdU was added. BrdU analysis can be performed in two manners (1) BrdU is added immediately after cell cycle entry and is incorporated into the DNA for several hours before cell fixation, staining and FACs analysis or (2) BrdU is added at various time points only for a short period of time before cell fixation. In the first case, BrdU incorporation is a measurement of the percentage of cells that have engaged in cell cycle entry immediately after serum stimulation and until the time of fixation, thus giving an overview of the total number of

cells undergoing cell cycle progress. On the other hand, in approach (2), BrdU incorporation occurs briefly and only to cells that undergo S phase at the moment of BrdU addition and not the total amount of cells that have undergone G1 to S transition. In this study the second approach was used where BrdU incorporation reflects the number of cells in S phase momentarily and throughout the whole time course. This approach was selected in order to evaluate at which time point TRAP 5a effect takes place without masking the effect from the accumulation of cells in the S phase.

The selection of BrdU for studying proliferation was based on two reasons. Firstly, BrdU incorporation in combination with propidium iodide (PI) can give a clear separation of the cell cycle stages G1, S and G2/M thus allowing for a detailed comprehension of the cell cycle and TRAP 5a effects. Second, viability assays broadly used for proliferation studies (e.g. XTT assay) are based on mitochondrial activity. Since TRAP 5a effects on cells are under investigation, it cannot be excluded that TRAP 5a alters the mitochondrial activity of the cells thus affecting the read-out of assays based on this type of activity.

3.6 PHOSPHORYLATION SIGNALLING ASSAYS

In paper IV, in order to investigate signaling assays triggered by TRAP 5a upon cell cycle entry, a signalling array detecting alterations in phosphorylation status was used. Since phosphorylation status of proteins involved in the cell cycle regulation occur very fast, the time points selected ranged between 5-30 min.

4 RESULTS AND DISCUSSION

4.1 PAPER I

In this study the aim was to develop a human TRAP isoform 5a- specific ELISA that would give the possibility of studying TRAP 5a levels in serum. Here, obese vs. lean individuals was compared in order to assess if TRAP 5a correlates to BMI in serum as previously reported [193].

Firstly we developed monoclonal antibodies (mAbs) specific for TRAP 5a using the loop present in TRAP 5a but absent in TRAP 5b due to proteolytic cleavage [131] as well as mAbs detecting both isoforms using the full length protein. Epitope mapping allowed grouping the mAbs according to the recognition epitope. Three groups of mAbs were identified for total TRAP (TRAP 5a+5b) one of which was mAb with the peptide loop as recognition epitope (mAb 46). Results showed that TRAP 5a could successfully be recognized by the specific mAb 46 both in Western immunoblot and ELISA and that a mAb (clone 12.56) raised against full length TRAP could recognise both isoforms. With the combination of a mAb against TRAP 5a (mAb 46) as capture antibody and a mAb against total TRAP (mAb 12.56) as detection antibody, the development of an ELISA was possible. Even though, antibodies specific for TRAP 5a have been developed and used in the past [194,195], this is the first time that mAb against TRAP 5a has been raised using the peptide loop present only in TRAP 5a, instead of subcloning which could potentially increase the specificity of an ELISA since it is assured that the recognition epitope is only present on isoform TRAP 5a.

After evaluation of mAbs, the ELISA was validated for isoform specificity using TRAP 5a and TRAP 5b isoforms and it was shown that with the combination of antibodies mAb 46 (TRAP 5a) and mAb 12.56 (TRAP 5a+5b) only TRAP 5a was detected and absorbence was dose-dependent with a lower detection limit of 0.112 ± 0.039± ng/ml. Since TRAP has previously been shown to associate with α2-macroglobulin in serum [167], the effect of serum in the dose-dependent response for TRAP 5a was evaluated by the use of commercially available serum samples. Hence it was shown that serum has no effect on TRAP concentrations up to 0.5ng/ml in the standard curve. At higher TRAP 5a concentrations, serum had an inhibitory effect on the absorbance which could be due to failure to completely dissociate complexes of TRAP 5a with serum components.

Inter- and intra-assay variation of TRAP were evaluated in a set of commercially available serum samples (3 samples for intra-assay and 17 samples for intra-assay) from healthy males. Intra-assay variation was calculated in average to 10.3% and inter-assay variation to 10.7% that are both in acceptable ranges for an ELISA assay. Apart from variations, the reference interval was calculated to 0.86-6.27 ng/ml while the mean concentration was 3.65±1.23 ng/ml. The mean concentration for healthy males is in the same range as reported previous studies where TRAP 5a was measured and have shown that mean TRAP 5a concentration in serum of healthy males was 2.65 ± 0.29 ng/ml [193] and 5.89 ± 1.65 ng/ml [195] in different groups of healthy males.

After validation of the newly developed ELISA, TRAP 5a ELISA was used to measure the levels of TRAP 5a in serum from lean vs. obese females. The same groups were used to measure total TRAP with a previously described ELISA [192] in order to evaluate if the relationship between the groups is the same for TRAP 5a and total TRAP. The mean concentration for TRAP 5a of lean individuals was calculated to 3.78 ng/ml and to 1.97 ng/ml for obese females using the newly developed TRAP 5a ELISA indicating that TRAP 5a levels were lower in obese individuals compared to lean. These findings seem to be in contrast with what has been observed in a previous study where TRAP 5a was elevated with increased BMI [193]. However in this previous study the group investigated was overweight and male rather than obese (BMI=25-30 kg/m^2) while in this study all individuals in the female obese group had a

BMI of over 30 kg/m^2. The same relation between lean and obese group was also indicated when total TRAP was measured, where the mean concentration for the lean group was 1.19 ng/ml and for the obese group 0.79 ng/ml. Despite the fact the two assays seem to decline in terms of absolute values, they seem to exhibit the same relation for TRAP levels between lean and obese group.

Finally, it was investigated whether there is a correlation between TRAP 5a and total TRAP levels with BMI for the group of lean and obese individuals. A significant correlation was only observed between TRAP 5a and BMI in the group of obese individuals, thus indicating that in lean individuals, multiple factors apart from the adipose tissue burden can contribute to the levels of TRAP 5a. In the study by Shih et al. in 2010 [193], correlation was observed in a group of individuals with BMI of 18.5-30 kg/m^2 however no separation between lean and overweight group was performed.

In summary, in paper I the development and validation of a new ELISA for detection of TRAP 5a isoform is described, with the use of monoclonal antibodies raised against the peptide loop present in TRAP 5a but not TRAP 5b (for TRAP 5a specific mAb) or the full length protein (for total TRAP mAbs), its evaluation using serum from healthy males and its use to detect TRAP 5a levels in serum from obese and lean females. Results showed a decreased TRAP 5a expression in the serum of obese (BMI≥30 kg/m^2) vs. lean females and correlation between TRAP 5a and BMI only in the obese group, perhaps indicating that only in obesity TRAP 5a levels are mainly determined by adipose tissue. It has also been shown that expansion and morphology of adipocytes in different depots (hyperplasia vs. hypertrophy) plays a role in the metabolic profile [109] and also that TRAP highest expression shifts from visceral in lean to subcutaneous in obese individuals [98], thus indicating that the depot and morphology of adipose tissue could also influence the serum levels.

4.2 PAPER II

The aim of this study was to identify potential binding partners for TRAP 5a in the adipose tissue and to investigate if the previously suggested binding partner for TRAP, i.e. TRIP-1 [152,165] is interacting with TRAP 5a in pre-adipocytes. For this purpose, cell membrane proteins from pre-adipocytes were isolated and used in a pull-down assay were His-tagged TRAP 5a was used as 'bait'.

After pull- down assay, 3 bands with molecular weight higher than TRAP 5a were observed by SDS-PAGE and silver staining and were analysed using mass spectrometry. All three bands contained a peptide sequence corresponding to protein nidogen-2. This finding was initially surprising since nidogen-2 is not known to be found on the membrane of cells, however, this could be due to nidogen-2 attaching to the cell surface via its RGD-region [196] and thus being isolated together with membrane proteins. Nidogen-2 has originally been shown to be an ECM protein and to have a high homology with nidogen-1 [197] while its expression is up-regulated during adipocyte differentiation [52], thus it was hypothesized that nidogen-2 could bind TRAP 5a after secretion of the latter from macrophages in the ECM in an interaction module similar to what has been observed for growth factors e.g. for HGF interaction with vitronectin and fibronectin [198].

Firstly, nidogen-2 mRNA and protein expression in pre-adipocytes and differentiating pre-adipocytes was investigated and it was shown that indeed mRNA expression of nidogen-2 is up-regulated during differentiation. Nidogen-2 was detected both in media and cell lysate of pre-adipocytes, however a large fraction was of lower molecular weight than full length nidogen-2, indicating a proteolytical processing event in one of its cleavage sites [199].

Localization of nidogen-2 in pre-adipocytes was investigated by immunocytochemistry and this protein was shown to be located in cytoplasmic vesicles and fiber-like structures, while in previous studies nidogen-2 was observed pericellularly [199]. Since identification of nidogen-2 by mass spectrometry had very low coverage, additional methods were used to verify if indeed it interacts with TRAP 5a. Pull down-assays were used with either His-tagged TRAP 5a or His-tagged nidogen-2 as 'baits' and untagged nidogen-2 or untagged TRAP 5a respectively. Nidogen-2 contains three distinct domains G1, G2 and rod-G3 with different binding motifs [197] and pull- down assays with the different domains were also used to evaluate which domain of nidogen-2 mediates the binding to TRAP 5a. Results showed that TRAP 5a could pull down nidogen-2 and that nidogen-2 rod G3 domain was responsible for the interaction between TRAP 5a and nidogen-2, thus further verifying that the potential interaction between the two proteins. Since nidogen-2 was shown to be localized to vesicle- and fibre- like structures in pre-adipocytes, it was investigated whether there existed a potential interaction of nidogen-2 with TRAP 5a. For this purpose, proximity ligation assay was performed in pre-adipocytes that had been treated with TRAP 5a, a showing that TRAP 5a and nidogen-2 are indeed interacting in pre-adipocytes. The interaction between nidogen-2 and TRAP 5a could occur in endocytic vesicles by endocytosis of TRAP 5a from the ECM, in complex with nidogen-2 fragments. Finally, the localization of TRAP 5a in relation to nidogen-2 in adipose tissue was investigated and it was shown that the two proteins are co-localized in vesicles in adipose tissue cells, indicating a potential interaction.

Apart from the interaction of nidogen-2 and TRAP 5a, the previously suggested binding partner for TRAP 5a, i.e. TRIP-1 was investigated for interaction with TRAP 5a in pre-adipocytes. This showed that TRIP-1 and TRAP 5a interact in vitro in a pull- down assay system and intracellularly in pre-adipocytes. Interaction between the two proteins intracellularly in vesicles was surprising since TRIP-1 has been suggested to be localized in the cytoplasmic matrix and nucleus [200] while TRAP 5a is not expressed by pre-adipocytes but only endocytosed and thus most likely located in endocytic vesicles.

In summary, this study aimed to identify new binding partners for TRAP 5a in pre-adipocytes and to investigate the previously suggested interaction of TRAP with TRIP-1 with the use of pull down and proximity ligation assays. Results revealed nidogen-2 as a potential binding partner of TRAP 5a intracellularly in pre-adipocytes and in adipose tissue and indicated that in pre-adipocytes TRAP 5a can interact with TRIP-1 intracellularly.

4.3 PAPER III

The aim in this paper was to determine if TRAP 5a can interact with the cell surface of pre-adipocytes and to investigate the routes through which TRAP 5a gets endocytosed by the cells. We also aimed to investigate TRAP 5a interaction with heparin and heparan sulfate and with the HPSG glypican-4 that has previously been suggested to interact with TRAP in bone [164].

Firstly, pre-adipocytes were treated with TRAP 5a and TRAP 5b at 4°C to prevent endocytosis and using immunocytochemistry and electron microscopy it was shown that TRAP 5a but not TRAP 5b can bind to the plasma membrane of the cells in a dose- dependent manner as shown by FACS. The staining revealed a spot-like pattern that could indicate the formation of receptor clusters, a process known to be used by some receptors for activation [201]. When the same method was applied at 37°C where endocytosis is allowed, TRAP 5a was shown to be endocytosed in a dose- dependent manner. Interestingly, no TRAP 5a endocytosis was observed for differentiated adipocytes, indicating that the expression of a binding partner for TRAP 5a in pre-adipocytes is down-regulated during differentiation.

Using gel filtration chromatography and activity assays, it was shown that TRAP 5a forms complexes with heparin and heparan sulfate, however only heparin was able to inhibit TRAP 5a phosphatase activity. The binding of TRAP 5a to heparan sulfate is of particular interest since a lot of proteins that can have a mitogenic effect mediate their effect through binding to heparan sulfate in the ECM and cell surface, e.g. FGF2 binding to FGF2 receptor leads to dimerization that is regulated by binding to heparan sulfate [202–204].

Co-localization between Glypican-4 and TRAP 5a was addressed and it was shown that TRAP 5a was co-localized with glypican-4 on the cell surface membrane and on endocytic vesicles, indicating that TRAP 5a could potentially bind to the cell membrane using the HPSG glypican-4 as a co-receptor for signalling and/or endocytosis facilitation as observed for other glypicans and their ability to bind to growth factors and facilitate signalling from the cell surface [205,206].

As described above, TRAP 5a was shown to bind to pre-adipocyte cell surface and found in intracellular vesicles so next we aimed to investigate the endocytosis route of TRAP 5a. Proteins that act as growth factors or mitogens have been shown to follow to major pathways of endocytosis. i.e. the clathrin- or caveolae- mediated pathways that are both dependent on dynamin. Firstly we questioned if a dynamin inhibitor could prevent endocytosis and it was shown that TRAP 5a endocytosis is indeed dynamin- dependent. Subsequently, using clathrin- and caveolae- specific inhibitors it was shown that TRAP 5a endocytosis is caveolae-mediated, which is an endocytosis route used only by a few growth factors since most growth factors are endocytosed in a clathrin-dependent manner [207]. Interestingly, it has been shown that caveolae-mediated endocytosis is responsible for insulin signalling [208] and that triacylglycerol synthesis takes place in the caveolae [209], thus indicating that TRAP signalling could potentially act in a similar manner. However this could also depend on the concentration of TRAP 5a used, as in this study 100M were used- a quite excessive concentration and it has previously been reported that cell could use caveolae- dependent endocytosis in the presence of high concentrations of growth factors to target them to degradation, e.g. EGF that at low concentrations is endocytosed in a clathrin- mediated manner but at a high concentration instead by a clathrin- independent manner [10].

TRAP 5a was shown to co-localize with the late endosomal marker Rab-7 and by electron microscopy to be also found in multivesicular bodies. However, it did not co-localize with the lysosomal marker LAMP-1, indicating that TRAP 5a either does not end up in the lysosomes or that it is degraded rapidly thus it is not detected, unlike what has been observed for TRAP in osteoblasts that co-localizes with LAMP-1[156], probably due to the different routes followed by the two cell types for TRAP processing since TRAP is expressed endogenously in osteoblasts [149–153] but not in pre-adipocytes [138].

Finally, mesenchymal stem cells and pre-osteoblasts and fibroblasts (that are also of mesenchymal lineage) were studied for their ability to bind TRAP 5a and it was shown that pre-osteoblasts but not MSCs or fibroblasts had the ability to bind TRAP 5a. TRAP 5a binding by pre-osteoblasts is in line with previous findings that indicate that pre-osteoblasts are able to bind TRAP [153]. Apart from pre-adipocytes TRAP 5a was shown to bind to pre-osteoblasts but not fibroblasts or mesenchymal stem cells.

In summary, this study aimed to identify if TRAP 5a binds to cell surface on pre-adipocytes and the route of endocytosis thereafter as well as to investigate if TRAP 5a colocalizes with the previous suggested binding partner glypican-4 and interaction with heparin and heparan sulfate. Results showed that TRAP 5a but not TRAP 5b binds to the cell surface of pre-adipocytes, gets endocytosed in a caveolae- dependent manner and ends-up in late endosomes and mulivesicular bodies. Additionally it was shown that TRAP 5a forms complex with heparin and heparan sulfate and colocalizes with the HPSG glypican-4.

4.4 PAPER IV

The aim of paper IV was to investigate the effect of TRAP 5a exposure, mimicking a paracrine situation, on cell cycle, differentiation and migration of pre-adipocytes and to identify potential signalling pathways affected by TRAP 5a treatment in pre-adipocytes shortly after cell cycle entry.

Firstly, cells were incubated with serum- free media for cell cycle arrest and synchronization and then they were allowed to enter the cell cycle in the presence or absence of TRAP 5a. Results indicated an increased number of cells in the S phase after 12h (~50% more cells) and increased cyclin D1 expression.

Cyclin D1 expression is an important hallmark in cell cycle progression as it signifies the G1 to S transition [24] and is regulated by intracellular signals such as e.g. GSK3β inactivation [22,23] by activated Akt [40] or by Wnt- mediated signalling [26], thus next we aimed to screen for potential phosphorylation changes in proteins of the Akt signalling pathway using a protein phosphorylation array. Interestingly results showed a decreased phosphorylation in Akt and Erk sites responsible for its activation suggesting that perhaps the action of TRAP 5a on cell number regulation is not mediated via direct activation of Akt or that TRAP 5a leads to faster response by the cells and transient activating phosphorylations of Akt [20] to be already down-regulated already after 5 min.

On the other hand, GSK3β showed reduced phosphorylation that leads to its inactivation and subsequent β-catenin stabilization that could account for effects on e.g. cyclin D1 levels and anti-apoptotic genes [34,210,211]. Apart from Akt, GSK3β regulation is mediated via canonical and non-canonical pathways of Wnt signalling [210] that is also involved in cell cycle entry and apoptosis reduction [34] and thus could be a potential signalling pathway affected by TRAP 5a. Stat3 activating phosphorylation was shown to be increased by TRAP 5a treatment and Stat3 has been shown to have an effect on the proliferation of pre-adipocytes, cyclin D1 levels and anti-apoptotic genes [212,213]. After serum addition and cell cycle entry an up-regulation of phosphorylations leading to activation of pro-apoptotic proteins p53 and p38 in control cells was observed. Increased activity of pro-apoptotic proteins could be attributed to hypermitogenic stimulation[28]. Interestingly, p53 and p38 exhibited reduced activating phosphorylation and Bad exhibited increased inhibiting phosphorylation after TRAP 5a treatment, indicating a potential effect of TRAP 5a in genes regulating cell cycle arrest and apoptosis, a mechanism that could also be involved in the cell number regulation as the 'break' in cell cycle progression. Activating phosphorylation of HSP27 that is involved in cell cycle arrest and is a downstream target of p38 [214,215] was also observed after TRAP 5a treatment, further suggesting a p38- and p53- mediated effect of TRAP 5a in cell cycle arrest. When p38 is activated cyclin D1 is inhibited thus leading to cell cycle arrest, while p53 is increased leading to p21 activation that results in cell cycle arrest or p16, p14/p19 activation resulting in premature senescence [216–218]. In this system, cells have been serum- deprived and cell cycle-arrested, thus a reduced activation of p38 and p53 would likely indicate an increased evasion of the cell cycle arrest after serum stimulation.

Proteins p38, p53 and heat shock protein 27 (HSP27) have been shown to effect several biological events, such as migration [215,219–222], which can be also coupled to proliferation. This idea is contrary to previous beliefs that proliferation and migration are two separate events that cannot take place simultaneously [223,224]. Since TRAP has previously been shown to have an effect on the migration of osteoclasts [225] and migration and morphology of cancer cells [185], we investigated the effect of TRAP 5a on morphology, migration and protein expression of proteins related to migration adhesion and cell shape such as focal adhesion kinase (FAK), paxillin and actin. Morphological analysis and wound healing assay showed that TRAP 5a lead to less circular and more elongated cells and increased migration. Also, synchronized pre-

adipocytes were assessed for morphology after serum stimulation in the presence of TRAP 5a and it was shown that cells became larger at low concentrations of TRAP 5a but less circular and more elongated at high concentrations. Protein expression of FAK and paxillin was decreased after 8h of TRAP 5a treatment, whereas β-actin was decreased after 5h, indicating that apart from morphology, TRAP 5a could exert an effect on the adhesion pattern of the cells.

Finally, the effect of TRAP 5a in early differentiation of pre-adipocytes was tested and it was shown that TRAP 5a enhances lipid droplet acquisition already after 2 days of differentiation independently of stimulation by the PPARγ activator rosiglitazone. The mechanism through which differentiation is affected by TRAP 5a remains unknown, however since pro-apoptotic proteins have been shown to have an effect on pre-adipocyte differentiation and differentiating pre-adipocytes exhibit increased resistance to apoptosis [226] it is likely that pre-adipocyte differentiation is stimulated by TRAP 5a via the same signalling pathway responsible for augmented G1 to S transition.

In summary, this study aimed to investigate the outcome of TRAP 5a treatment on pre-adipocytes and it was shown that TRAP 5a enhances the proliferation, migration and differentiation of pre-adipocytes. In a protein level, TRAP was shown to increase cyclin D1 levels and to change the phosphorylation pattern of GSK3β and to decrease activating phosphorylation of pro-apoptotic proteins p53 and p38. Finally, TRAP 5a was shown to affect cell morphology and reduce the expression of β-actin, FAK and paxillin. These results suggest an overall effect in the cell cycle events by TRAP 5a that could possibly be mediated by a common pathways affecting GSK3β activity.

4.5 GENERAL CONCLUSIONS AND DISCUSSION

In paper I it was shown that serum levels of TRAP 5a from obese females are lower than those from lean females. As discussed earlier, this was an interesting finding since TRAP levels in adipose tissue of obese women are elevated [192]. This could indicate different processing mechanisms of TRAP 5a from adipose tissue in lean and obese states. TRAP 5a expression is increased but perhaps the deposition to the serum could be reduced and more TRAP 5a remains associated to the tissue where it can stimulate the proliferation and differentiation of pre-adipocytes. Also since TRAP 5a has been shown to be endocytosed by pre-adipocytes in paper II, TRAP 5a levels in adipose tissue could be maintained high by endocytosis in the tissue. It has been shown that the size of adipocytes and type of obesity (hyperplastic vs. hypertrophic obesity) in visceral and subcutaneous depots is important for the expression levels of cytokines as well as for the metabolic effects [109], so the depot and type of obesity could also be related to the TRAP 5a levels in serum. Decreased levels of tissue in the serum of obese women seems also contradictory to a study performed in Chinese overweight males where it was shown that TRAP 5a is elevated in males with BMI 25-30 kg/m^2[193]. However, as discussed earlier the study of paper I differs from the previous study in the BMI grouping (study I was performed in a group of obese females with BMI ≥30 kg/m^2) as well as sex. It is therefore possible that TRAP 5a levels increase in serum of overweight individuals but decrease in the obese state which is more severe.

TRAP 5a is reduced in the serum of obese females but increased in the adipose tissue. In paper II and paper III it was shown that TRAP 5a can bind to components of the adipose tissue such as nidogen-2 and heparan sulfate. As discussed earlier, proteins with mitogenic effects are often binding to ECM proteins to be delivered to the cells or to be stored until they are needed when ECM is degraded and they are released. Since TRAP 5a seems to have an effect on proliferation, differentiation and migration of pre-adipocytes, thus exhibiting growth factor properties, the biological implications of such interactions with components of the ECM could

serve the purpose of storage and later release by degradation of the ECM, or as to be delivered to the cells for binding on the cell surface.

Binding of TRAP 5a on the cell surface is accompanied by co-localization with the HPSG glypican-4, also suggesting that if the mitogenic effects of TRAP 5a are following a mechanism of action similar to growth factors, glypican-4 could act as a co-receptor for TRAP 5a binding. Glypican-4 has been shown to enhance the signalling of Wnt 5a and to get endocytosed together with Wnt receptor Frizzled-2[227], which is consistent with the observation in paper III of co-localization of TRAP 5a with glypican-4 intracellularly and on cell surface, thus suggesting that the endocytosis and effect of TRAP 5a could potentially also be mediated in a similar manner as Wnt proteins. This hypothesis could further be strengthened by results from the signalling array in paper IV where reduced phosphorylation leading to inhibition of GSK3β activity was observed after TRAP 5a exposure and as discussed previously, GSK3β activity is mainly regulated by Akt and Wnt (canonical and non-canonical) signalling. Endocytosis of TRAP 5a occurs via the caveolae pathway and even though, generally caveolae pathway is a rare route for signalling process but rather for degradation it is responsible for the insulin endocytosis and triacylglycerol synthesis in adipocytes [208,209] indicating an importance in adipocyte signalling. Binding of TRAP 5a was also observed for pre-osteoblasts, which is similar to what has been shown in previous studies for endocytosis of TRAP and effect on their differentiation [151,153,187].

TRAP 5a was also shown to affect p53, p38 and Bad phosphorylation pattern indicating that there might be a potential regulatory role of TRAP 5a in cell cycle arrest/ senescence signalling. These data suggest that TRAP 5a could affect both the 'gas' and 'break' mechanisms of cell cycle progression.

On cellular level TRAP 5a leads apart from increased proliferation to increased migration as well as differentiation which in normal tissue could be important for tissue repair. On the other hand in pathological situations, dysregulation of TRAP expression could potentially be responsible for cell transformation to a more proliferative and migrating phenotype as observed for cancer cells with increased TRAP levels.

4.6 REVISED WORKING HYPOTHESIS

In light of the data in this thesis and previous studies on TRAP 5a action in adipose tissue, a revised hypothesis of TRAP 5a action is described in figure 9. A possible mechanism of action would be that TRAP 5a is secreted by a subset of macrophages and interacts with components of the ECM such as HSPGs and nidogen-2 in order to be stored or delivered to pre-adipocytes in close vicinity. TRAP 5a on the cell surface of pre-adipocytes could bind to a yet unidentified receptor either directly or indirectly facilitated by glypican-4 and HSPGs. TRAP 5a endocytosis via the caveolae- mediated pathway and presence in multivesicular bodies and endosomes could trigger a signalling pathway mediated by changes in phosphorylation of GSK3β, leading to various possible outcomes, such as increased cyclin D1 expression and G1 to S transition and migration and/or differentiation to adipocytes. These outcomes would be relevant for tissue homeostasis in a tissue repair/ wound healing situation. If dysregulated, eg overexpression, these outcomes could potentially lead to cell transformation.

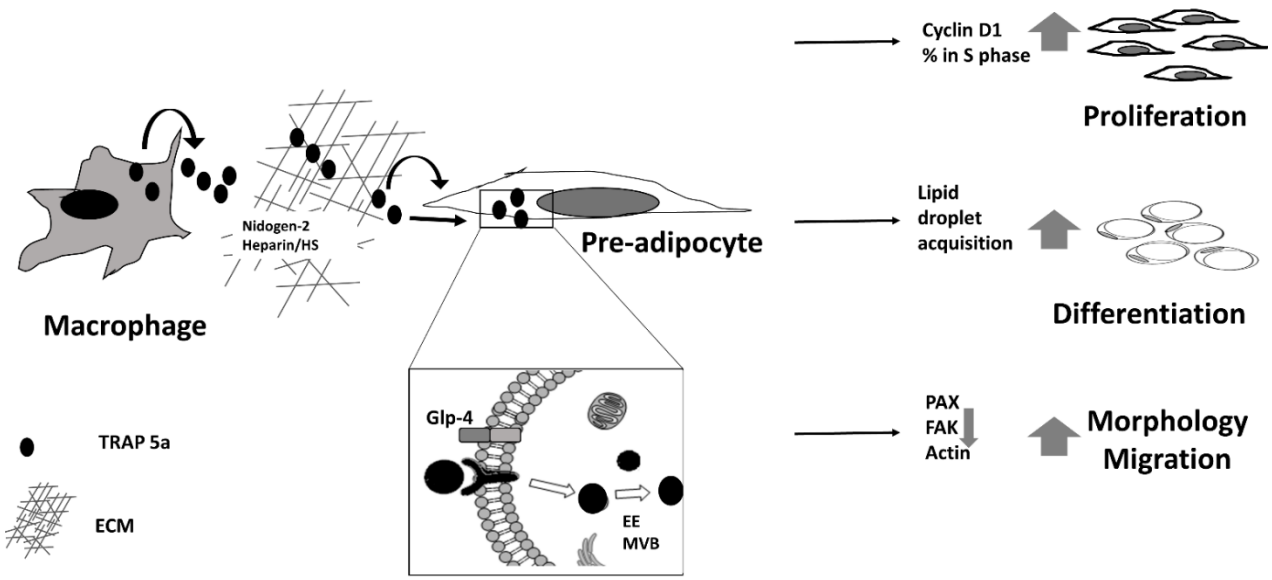

Figure 9. Revised working hypothesis. TRAP 5a secreted from macrophages in adipose tissue binds to the ECM probably by interaction with nidogen-2 and heparin or heparan sulfate. TRAP 5a binds to the surface of pre-adipocytes in proximity to the HSPG glypican-4 and is endocytosed via the caveolae pathway. Intracellularly it is found in early endosomes and multivesicular bodies but is not co-localized with LAMP-1. TRAP 5a enhances the proliferation of pre-adipocytes and increases cyclin D1 levels, increases differentiation (assessed by lipid droplet acquisition) and causes morphological changes and migration enhancement and decrease in FAK, paxillin and actin.

With regard to this hypothesis the following question would be relevant: How can such an action from a macrophage- secreted molecule contribute to tissue homeostasis? TRAP 5a mutations in humans have been shown to lead to bone dysplasia and autoimmune disorders, however there have not been any published observations of complete lack of adipose tissue formation [174,177], thus indicating that TRAP 5a is not critical for embryonic adipose tissue development. An alternative explanation could be that TRAP 5a contributes to tissue homeostasis in mature adipose tissue in cases where tissue repair or regeneration is needed. As previously mentioned, adipose tissue exhibits a stable number of adipocyte cells and an annual turnover of 10% [55,56], however in obesity there is an increased rate of adipocyte cell death and increased number of macrophages [98], suggesting an increased need for tissue 'repair'. Thus, one explanation of the biological relevance of TRAP 5a action would be as a part of the tissue repair (a wound healing-like action) of adipose tissue in obesity, triggered by macrophages. Similar mechanism have been previously shown for macrophages in other types of tissue such as skin where M2 anti-inflammatory macrophages express growth factors and stimulate cell differentiation and tissue repair in injury sites [96] and TRAP 5a could potentially be a part of the tissue repair mechanism in adipose tissue. However, several aspects of this hypothesis need to be further investigated.

4.7 FUTURE PERSPECTIVES AND CLINICAL SIGNIFICANCE

The revised hypothesis on TRAP 5a action presented above, would indicate several areas of research regarding TRAP 5a action.

Up to today, the subset of macrophages that express TRAP remains unknown and as previously described, macrophages can adopt a pro-inflammatory (M1) or anti-inflammatory (M2) phenotype as well as intermediate stages. Identification of the phenotype that expresses TRAP 5a would further contribute to the understanding of the role of TRAP 5a in adipose tissue. Additionally stimuli of TRAP expression from macrophages e.g. hypoxia, free fatty acids etc. that participate in macrophage recruitment and activation in adipose tissue remain to be investigated. Moreover the finding that TRAP 5a serum levels are reduced in the obese state, could be further studied by correlating TRAP 5a levels to type of obesity (e.g. hyperplastic or hypertrophic), adipocyte size and number. The newly developed TRAP 5a ELISA could be used to further investigate the use of this specific isoform as a marker for disease.

The difference between the functions two isoforms is of particular interest as it represents a situation where proteolytical cleavage switches the function from a protein with mitogenic/growth factor-like effect (TRAP 5a) to a phosphatase (TRAP 5b). Thus the conditions under which this transition occurs would be of interest in future studies.

Regarding TRAP 5a action in pre-adipocytes, one important future aspect would be the identification of the signalling binding partner of TRAP 5a on the pre-adipocyte cell surface. Identification of this binding partner would allow for further targeting of TRAP 5a action and future use for screening in other tissues for similar action.

Additionally, the exact signalling pathway of TRAP 5a in pre-adipocytes remains to be identified. A future study could involve the testing of hypothesis of wnt signalling- mediated TRAP action by the use of wnt signalling receptor inhibitors.

Finally the hypothesis of TRAP 5a acting as a part of tissue repair mechanism could be further tested in other tissues of mesenchymal origin. Studies suggest similar effects in pre-osteoblasts thus the effect of TRAP 5a on chondrocytes could also be of interest.

In summary, further elucidation of TRAP 5a mechanism action by identification of the binding partner and signalling pathway could contribute to the development of a drug target for hindering increased proliferation, migration and differentiation of pre-adipocytes and thus adipose tissue development. Moreover, TRAP 5a mechanism of action could be useful for the development of methods to promote adipose tissue regeneration in pathological situations such as cachexia where absence of adipose tissue can be life threatening. Additionally, it is of importance to investigate if the mechanism applies to other normal tissue types such as bone and cartilage or in cancer tissues where TRAP has also been shown to play a role.

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